

Republicans foster science (*pro tem*)

After a year, the Republican party has full control of the US Congress and has been kinder to science than its critics allow. But that disposition is unlikely to last for long.

A YEAR ago this week, when the Republican Party won control of both houses of the US Congress for the first time in 40 years, few anticipated how quickly they would come to dominate the Washington agenda. There is, after all, still a Democrat in the White House. But the growing fissure between Clinton and his alleged Democrat colleagues in Congress has disarmed the Democrats and in science policy, as elsewhere, the Republican hegemony is virtually complete.

The Republicans have been kinder to science than the science lobby cares to admit. Although the budget for the present fiscal year, which started on 1 October, has yet to be agreed, the National Institutes of Health are the only substantial component of it for which the House of Representatives, the Senate and administration each call for an increase that will cover inflation. The National Science Foundation and the main physics accounts at the Department of Energy are set to get flat funding. Sharp cuts will be made in fusion research, in the search for renewable energy sources, in environmental research and in technology support programmes. It will not comfort the victims of these cuts to point out that, in the case of fusion and renewables, the reductions are merely the logical outcome of years of programme drift at the Department of Energy, and that the environment and technology programmes are simply being returned to where they were before Clinton's election.

But in the longer term, the picture for science funding does not look bright. Given that science and technology account for one-seventh of the entire discretionary budget, that is inevitable. Budget projections being prepared by the Republicans for the next seven years generally assume flat funding (whose value would be eroded by inflation) for most science agencies, and sharp cuts at Energy and at the National Aeronautics and Space Administration (NASA). But these are approximations; actual programme budgets will be set by the annual appropriations process.

Democrats have argued that all of this amounts to a wholesale onslaught on the tradition of the enlightenment. But their arguments have been undermined by the inconsistency, on budget matters, of the Clinton administration. To recap briefly, the administration proposed a budget last February that was generous to most science programmes, but would have slightly increased the budget deficit. Administration officials defended that budget as prudent, because, they said, the deficit would increase by less than the growth in the overall economy. The administration would borrow more in

1996, in other words, but not much more. For domestic critics, and for the international financial markets that now dictate these things (see *Nature* 377, 562; 1995), this was clearly inadequate.

Acknowledging as much, Clinton hastily revised his budget in July, saying he would balance it after all. But details of how that would be achieved, on a programmatic level, have never been spelt out. The administration has budget figures for its science agencies — the February ones — but it has none to match the president's recent rhetoric, as he has crawled to meet the Republican position. Thus the initiative has not so much been grabbed by the Republicans as been thrust upon them.

So what have they done with it? Bob Walker (Republican, Pennsylvania) did not much want to chair the House Science committee, but he has emerged as an honest and effective leader for science in Congress. His approach to running his committee verges on the dictatorial. But the committee's output counts for something, and everyone operating under its jurisdiction — including most people in the physical sciences in the United States — knows where to complain.

The life sciences, meanwhile, have looked to old Republican allies for preservation, and have not been disappointed. Even Newt Gingrich (Republican, Georgia), the House speaker and Washington's dominant figure, has been convinced that biomedical research is deserving of strong support from the federal government.

But Gingrich, addressing the American Association of Medical Colleges (AAMC) last week, made it pretty clear that such support is unlikely to extend to inflation-matching increases each year (see page 117). Citing indices which show that some services are becoming cheaper each year because of improvements in efficiency and technological change, he told the medical schools to start thinking about doing more for less. It may be time for research providers to start addressing this argument.

Gingrich's impulsiveness scared some of the 2,000 AAMC delegates, while others were impressed with his clarity of purpose and, above all, his obsession with the power of ideas. Senator Christopher Dodd (Democrat, Connecticut), chairman of the Democratic National Committee, who had the misfortune to follow Gingrich at the podium, sounded like a stump politician, vainly seeking to defend the priorities and programmes of the past. The contrast between the two men spoke volumes about what is happening in Washington, one year into the Republican revolution. □



Advisors may urge NIH to seek out industry support for clinical trials

Washington. A panel of advisors to the National Institutes of Health (NIH) is expected to recommend that the NIH should investigate ways of obtaining financial and other support from the medical insurance and pharmaceutical industries for its clinical studies, in a bid to counter the escalating cost of such research.

The NIH's \$1.7-billion clinical research programme has been under review since July by the Panel on Clinical Research, whose 15 members include administrators of leading research institutions and senior industry officials, and which reports to Harold Varmus, the director of NIH.

Its full report is not due until next year. But at a meeting of the panel in Bethesda, Maryland, last week, members revealed that they are studying a number of ways in which the private sector could "partner" the NIH in its support of clinical research.

The panel's chairman, David Nathan, president of the Dana Farber Cancer Institute in Boston, said he wants the group to recommend that medical insurance and drug companies contribute an annual amount to NIH towards the cost of large clinical trials, from which they often benefit.

"The taxpayer should not be paying the

cost alone," Nathan said. But he emphasized that, even if industry did contribute, it would not decide what research was carried out; indeed, Nathan said any one company might or might not benefit from such research in any particular year, just as certain taxpayers gain from federally funded research conducted in one year and not another.

He added that conflicts of interest would be largely avoided because the grant from industry would be administered by the NIH, and that the trials would, as at present, be carried out by independent investigators.

Nathan said that the NIH has reached a point at which it either has to reduce the number of clinical trials it funds or to raise outside contributions to the costs of the trials. Any extra money received from industry would release funds for spending on areas of clinical research in which "we have to beg for money now", Nathan added.

It remains to be seen how many of the other panel members will eventually back this approach. But there is already caution from one representative of the pharmaceutical industry. Leon Rosenberg, a member of the NIH advisory panel who is president of the Pharmaceutical Research Institute of Bristol-Myers Squibb, said the idea "that a

bunch of pharmaceutical companies would bond together to supplement an NIH appropriation is not very likely to come to pass". (The Pharmaceutical Manufacturers Association says it does not want to comment before it has been able to study detailed proposals.)

Rosenberg did say that drug companies should become more involved in training young clinical researchers, who would benefit from seeing "ideas get converted into drugs or devices that help the public". He also suggested that this might avoid potential conflicts of interest. "I can't imagine a postdoc in oncology would find a conflict in learning about study design and data management at Bristol-Myers," he said.

Others panel members expressed worries over the conflict-of-interest implications of Nathan's suggestion. And there is likely to be similar concern in Congress. Chris Shays (Republican, Connecticut), for example, chair of a House of Representative subcommittee with some oversight responsibility for the NIH, says he can see the value of asking industry to help cover the costs of clinical trials. But he is concerned that conflicts of interest might arise from attempts by industry to exert influence on research they are asked to support. "The studies must be independent," he says.

The panel also discussed whether industry should be involved in NIH's 75 General Clinical Research Centers, run jointly by NIH and academic institutions under a programme that provides clinical research facilities and personnel to scientists without other support. Nathan pointed out a possible model in a thriving venture between the Massachusetts Institute of Technology, Beth Israel Hospital and the drug company Pfizer.

But some panel members believe that, if industry is to become a major funder of NIH research, it will be necessary to ensure that universities, which at present rely on drug companies for a substantial amount of support, are not left out.

The major question remains whether corporations will be willing to contribute. "Will directors feel they can give lower dividends to stockholders in exchange for research which may benefit them in the long term?" asked Nathan. He made clear his own view that the stockholders would benefit from such investment.

Other issues being considered by the panel include whether NIH's clinical research programme should pay physicians more, the impact of the growth of managed care, with its emphasis on cost-cutting, and whether the United States is training enough clinical researchers. **Adrianne Appel**

Hubble reveals glory of Eagle's 'EGGs'

London. The orbiting Hubble Space Telescope (HST) has recorded the birth of new stars in the Eagle nebula, 7,000 light-years from Earth in the Serpens constellation. The towering dark structure in the picture (right) is a column of cool molecular hydrogen and dust that acts as an incubator for the infant stars. The gas is slowly eroded by ultraviolet photoevaporation from nearby hot stars to uncover small globules of a dense gas. These evaporating gaseous globules have come to be known colloquially as 'EGGs'.

The shadows of the 'EGGs' protect gas behind them, resulting in the projections at the top of the cloud. Forming inside at least some of the 'EGGs' are embryonic stars — which abruptly stop growing when the 'EGGs' are uncovered and separated from the larger reservoir of gas from which they were drawing mass.

The picture, taken with the HST's

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Wide Field and Planetary Camera 2, was constructed from three separate images representing emission from different types of atoms. The red regions of the picture show emission from singly ionized sulphur atoms. Green indicates emission from hydrogen and blue shows light emitted by doubly ionized oxygen atoms. □

Space Telescope Science Institute

Senate bill seeks to tighten controls on foreign researchers

Washington. An immigration reform bill introduced in the US Senate last week would, if passed in its current form, sharply reduce the number of foreign professional workers allowed into the country each year, and require non-US students to return home for at least two years after obtaining their degrees before being allowed to work in the United States.

The bill, sponsored by Senator Alan Simpson (Republican, Wyoming), would also force employers to pay substantially more to employ non-US than American workers, which its critics fear could discourage the hiring of foreign researchers.

The Simpson bill joins a bill already moving through the House of Representatives as the main vehicles for immigration reform in the current Congress (see *Nature* 376, 373; 1995). Of the two, the Senate bill is more restrictive. Total employment-related immigration would drop from 140,000 a year to 90,000 a year — roughly the number admitted last year, not counting unskilled workers and Chinese students, who receive special consideration. The House bill would set the ceiling at 135,000.

Under Simpson's bill, US employers would have to pay foreign-born workers at least as much as American workers. In addition, however, a further 25 per cent of the worker's salary would have to be paid by employers into a fund for training US workers. Temporary professional workers (those holding H-1B, H-2B and L visas) would be allowed to stay for only three years, half of the period currently allowed. Their employers, too, would have to contribute up to 22.5 percent of the foreign employee's salary into a training fund.

Perhaps the most controversial clause for the university community will be the bill's stipulation that work visas will be granted only to those who have worked at least two years outside the United States after receiving their most recent degree — effectively barring foreign students from staying on immediately after graduation. This requirement, says Simpson, "is intended to provide protection for US workers who are just beginning their careers".

Opinion polls consistently show that US voters favour tighter restrictions on immigration. But businesses and universities tend to be opposed, and have lobbied successfully to soften the harder edges of Smith's bill — including reinstating a special visa category for "outstanding researchers". The fate of Simpson's bill is therefore hard to predict, and will depend in part on how emotive the immigration issue becomes in next year's presidential election.

Tony Reichhardt

Managed health-care adds to woes of US medical schools

Washington. Medicare reform, the growth of managed health-care, and budget uncertainty at the National Institutes of Health (NIH) are combining into a powerful threat to both research and teaching at medical schools in the United States, academic leaders were told at a conference in Washington last week. "It is clear that funding from all sources is going to be severely constrained," said Jordan Cohen, president of the Association of American Medical Colleges (AAMC).

But Newt Gingrich (Republican, Georgia), the speaker of the House of Representatives, told the annual conference of the AAMC that the plight of the schools was appreciated in Congress. He announced his backing for plans for a 'trust fund' to replace the existing indirect flow of money from Medicare — the system that pays for health-care for elderly Americans — into the teaching hospitals.

The fund, said Gingrich, would "put [the costs] on the table and educate the country about the importance of medical education, and why we as a government have a legitimate role in ensuring that it happens". But he added that the health-care system should not expect automatic increases in funding to allow for inflation.

Such indexing, he said, assumed no changes were being made to improve efficiency. "If you first set up a bureaucracy which is wasteful, fraudulent and incompetent, you then measure what that bureaucracy does, you then say that's the inflation rate because you can't get any smarter than the current bureaucracy," said Gingrich. "It becomes tautologically impossible."

Reform of Medicare and Medicaid — the smaller system that helps poor Americans of all ages — are part of the huge reconciliation budget bill that Congress will try to force President Bill Clinton to sign this month. Under the Republican plans, the government would buy health-care plans for many patients, instead of paying for their health-care directly. The money would therefore go to health maintenance organizations (HMOs) rather than hospitals, which use as much as \$6 billion of it each year to subsidize their medical schools.

The Senate has now proposed a scheme that would take some of this money before it went to the HMOs, and redirect it to the

hospitals. The House, in contrast, proposes a trust fund supported by a direct congressional appropriation for medical education, growing to \$4 billion a year by 2002, to replace the existing indirect support. Biomedical lobbyists say both proposals would deprive the medical schools of more than \$8 billion in support between now and 2002.

The two proposals arrive at a time when teaching hospitals are suffering severe revenue loss because of the national trend away from generous health insurance to 'managed care' plans, which rigorously supervise how health-care money is spent.

Managed care is already dominant in some parts of the country, imposing shorter stays at cheaper institutions. According to George Mandel, chair of pharmacology at the George Washington University Medical Center in Washington DC, its impact has slashed the number of beds that the centre's hospital can support from 500 to 250.

Sam Silverstein, chair of physiology at Columbia University in New York, and past president of the Federation of American Societies for Experimental Biology (FASEB), a biomedical lobby group, estimates that up to \$1 billion of general income each year supports research in the academic medical centres. But it does not show up in their accounts, Silverstein says, because the rules for indirect cost recovery discourage its declaration. Silverstein says that, in New York, the impact of the managed care revolution has still to be felt.

Apart from these indirect threats, biomedical researchers this month face the possibility of a severe squeeze on funding at NIH, their main funding agency. Five weeks into the financial year, the NIH, like most of the rest of the government, has no budget agreed. Until 13 November, it will operate under a continuing resolution at 95 per cent of last year's funding level. After that, it may fall victim to a longer and even more severe continuing resolution. This week, NIH's political allies, led by George Gekas (Republican, Pennsylvania), will try to separate it from other elements in the federal budget, on the grounds that the House, the Senate and the administration all agree that it should get an increase in funding.

Most NIH grants start or resume at the beginning of December, and the NIH has written to all grant recipients warning them of the uncertainty. The nature of the extended continuing resolution will determine what they get in December. "We're looking at various different approaches," says Lee Pushkin, assistant director for budgets at NIH. Silverstein expects NIH to cut deeply into existing grants, if it must, to preserve the flow of new awards.

Colin Macilwain

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Gingrich: backs fund
for teaching hospitals.

EU sets new rates for young researchers...

Munich. The European Commission last week agreed new rules for assessing the value of fellowships awarded to young scientists under its Training and Mobility of Researchers (TMR) programme. The agreement, designed to bring awards more closely in line with local pay scales, comes just in time for the distribution of the first round of grants under the fourth Framework Programme, worth ECU41.4 million (US\$54.6 million), approved in September.

Until now, the commission has provided a fixed sum to support young scientists from

each European Union (EU) country undertaking research for up to two years in another member state. But different taxation rules and demands for accompanying overhead payments from host institutions have meant that the amount passed to researchers varied significantly between countries, and have often been grossly out of line with local salaries. In Germany, for example, a grant-holder often ended up being paid as much as the head of the department he or she was working in (see *Nature* 361, 196; 1993).

Recognizing the problems that this

caused, the EU council of ministers agreed last year to seek a mechanism to ensure that all TMR grant-holders receive the same net income as a national researcher at the same professional level. The commission has now approved rates for studentships and fellowships submitted by each member country that achieve this goal.

In most countries, awards will take the form of a taxable work contract, rather than a bursary. The net payment made by the commission will vary between about ECU900 and ECU1,500 a month for a studentship, and about ECU1,300 to ECU2,000 for a fellowship, depending on the host country. A single flat rate of ECU10,000 a year in overheads will be paid in each member state.

This system for distributing awards will be adopted as a model by all other EU research programmes. But, although more equitable than the previous system, it has its price. Under the new rules, TMR fellowships are more expensive, and the total number of grants that can be offered has therefore fallen by several hundred. But the commission still hopes to award up to 4,000 training grants with the ECU260 million it has to distribute over the next four years.

The commission also agreed last week that two-thirds of the total will be allocated as postdoctoral fellowships. Only 20 per cent will be given as studentships, in line with the commission's policy that graduate education should be a national, rather than a European, concern. Ten per cent of the money will be given to grant-holders returning to 'less privileged' regions of the EU, such as Merseyside in the United Kingdom, eastern Germany and some Mediterranean countries, on condition that they continue their research for up to a year.

Despite the much criticized bureaucracy of the mobility programme, its popularity continues to grow. More than 2,200 projects were submitted in the first call for proposals in summer, of which 513 are to be funded. While the success rate for fellowship applications was 28 per cent, the commission received significantly more applications for PhD grants than it had expected, pushing the acceptance rate to below 10 per cent.

Oversubscription has also caused a problem in the part of the TMR programme that funds research networks. Only 6.4 per cent of the proposals submitted earlier this year for the first call, involving the distribution of ECU150 million, could be accepted.

"We are not able to fund excellent projects, only outstanding ones, and we are very disturbed to see top-graded applications being turned down", Jürgen Rosenbaum, a spokesman for the TMR programme, told a meeting of German university administrators held in Regensburg last month.

Alison Abbott

...and continues funding for INTAS

Munich. European Union (EU) research ministers last week agreed to give the European Commission in Brussels greater control over the operations of INTAS, the intergovernmental organization that channels European money to basic scientists in countries of the former Soviet Union.

In return, the ministers agreed that the commission should provide sufficient support to allow INTAS to continue to operate for a further four years, and that it should remain an independent body, rather than being incorporated into the EU's general research programme for central and eastern Europe.

INTAS was established in 1993, on the initiative of the former French president, François Mitterrand, by the European Commission and the 15 current EU member states, plus Norway and Switzerland. Its goal is to help to support scientists in what are known collectively as the new independent states (NIS) of the former Soviet Union.

But its future had been put in doubt by criticism from parts of the scientific community, subsequently taken up by the new research commissioner, Edith Cresson, of the way its internal administrative procedures led to long delays before funding for approved projects reached the NIS scientists concerned (see *Nature* 374, 203; 1995).

This, in turn, had fanned discontent among member states. Most of the ECU53 million (US\$70 million) that INTAS has so far received has come from the commission's research budget. But ECU3 million has been provided by individual member states which are no longer so keen to provide additional funds.

Faced with the likelihood of becoming the sole contributor, the commission has considered dissolving INTAS and distribut-

ing the funds available for scientific cooperation with NIS through its existing international cooperation (INCO) scheme.

INCO has an annual budget of ECU60 million for research projects in central and eastern European countries and the NIS, although the commission has yet to agree how this sum should be divided between the two groups. But some member states opposed the plan to merge it with INTAS. As a result, the council of ministers has agreed that, in exchange for giving the commission more strategic control over its activities, INTAS should continue its independent existence, receiving half of the research funds allocated by the commission for research projects in the NIS (the other half will go on applied research projects).

Under the new terms, the commission will chair the INTAS assembly — the body that sets its general policy — and will be able to veto all its decisions. "This will ensure that we can promote synergy with other commission research programmes," says Rainer Gerold, a spokesman for the INCO programme.

The research ministers called for additional measures to make INTAS more efficient — including a reduction in the number of administrative staff at its headquarters in Brussels. They also called for individual member states to continue additional funding, and for research organizations of the NIS to increase their own contribution. This year, for example, Kazakhstan, Russia and the Ukraine jointly contributed ECU3.7 million to research projects carried out with scientists in Western Europe.

The commission will decide over the next few weeks how its total budget for supporting research in central and eastern Europe and the NIS is to be divided. Just over half is likely to go to central European countries, including Poland, the Czech Republic and Hungary, in recognition of their interest in EU membership. If this happens, INTAS will receive around ECU13 million a year over the remaining three years of the Framework programme.

A.A.

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Rev Features

UN climate change report turns up the heat

London. A report to the United Nations on the economic dimensions of climate change may be published with a key chapter missing unless a dispute can be settled between the chapter's authors and delegates from developing countries.

The chapter, which forms part of an overall study on the economic and social implications of climate change from working group three of the Intergovernmental Panel on Climate Change (IPCC), suggests that the costs required to slow down greenhouse gas emissions may exceed estimates of damage from climate change.

But the summary of the report, written explicitly for policy-makers and agreed by the working group in Montreal last month, effectively cancels this conclusion. Its writers argue that damage estimates would be higher if the chapter's authors had used the same criteria to assess losses in rich and poor countries, rather than, for example, estimating loss of life of an individual in a poor country at US\$100,000, one-fifteenth of the value in a rich country.

As a result, the authors of the chapter have decided to withhold their work unless they are allowed to respond to criticisms of their calculations made in the summary, which is written by experts from governments, particularly Cuba, India, Colombia and the Alliance of Small Island States.

"I would prefer to publish the chapter with an addendum making clear why we [the authors] disagree with the summary for policy-makers," says David Pearce, director of the Centre for Social and Economic Research of the Global Environment at University College London and the lead author for chapter six.

A senior IPCC official has said that any changes to the report's content should have been made during two earlier rounds of peer review among governments and independent experts. "At this stage, the authors can make a few editorial changes for clarity of reading, but not changes to the meaning or substance of the report," says James Bruce, co-chair of working group three.

Under IPCC rules, authors of chapters are responsible for overall editorial content. A chapter cannot be published by the panel unless all authors transfer their copyright to the climate body. One of the authors of chapter six, William R. Cline, a senior research fellow at the Institute for International Economics in Washington DC, agrees he would rather have the chapter erased from the IPCC's final report than see it included in its present form.

But although Bruce concedes that the omission of the contentious chapter "could cause problems" for the IPCC, news of the chapter's probable withdrawal is likely to be welcomed in many developing countries, as well as in the London offices of the Global

Commons Institute (GCI), a group of environmentalists behind the campaign to "rewrite or withdraw" the chapter.

GCI has successfully lobbied developing countries to call for a recalculation of the damage that would result from a doubling of carbon dioxide concentrations by the year 2050 in a way that would require richer countries to shoulder more responsibility for the effects of climate change.

The chapter's authors had valued the damage from climate change at 1.5–2 per cent of gross world product (GWP), the



market value of all goods and services sold throughout the world. But GCI argues in a paper written for *The Ecologist* that damage estimates would be higher — between 12 per cent and 130 per cent of GWP — if based on a formula that asked countries how much compensation they would be willing to accept for the losses from climate change.

When the authors refused to alter their calculations, GCI persuaded those responsible for the summary for policy-makers to erase references to damage estimates, and include phrases such as "the literature on the subject in this section is controversial", and "the value of life" and "the loss of

unique cultures" cannot be quantified.

But Pearce argues that the GCI formula "is not supported by published data", and would not necessarily have increased the estimates for climate change damage in the developing world. One of the few known research papers to use the 'willingness to accept' method — from the Indira Gandhi Institute of Science in Bombay — resulted in an estimated \$10,000 value for loss of life.

Pearce claims that GCI has tried to turn an essentially scientific process into a political one. He says it should not have interfered with the process of independent scientific inquiry. "The IPCC is not a policy-making body. It is a body of scientific experts. We had strict instructions from the IPCC only to review the existing literature and not create any new literature."

But Aubrey Meyer, the director of GCI, disagrees and says Pearce and his team "are in no position" to label others with the charge of compromising scientific objectivity. Four out of the seven authors of the chapter, says Meyer, are the most frequently recurring names in the chapter's list of references. The same four names, he adds, form the majority of references linked to parts of the chapter dealing with damage estimates. "How can this be an objective process when the authors spent much of the time reviewing each other's work?" he asks.

Meyer says that the rule that authors cannot create literature should be lifted. He adds that the composition of authors should also better reflect the world's demography; only two of the chapter's seven authors were from the developing world.

But Pearce claims that Meyer and his colleagues have behaved "irresponsibly" in seeking to have quantitative references to damage estimates removed from the summary, as "99 per cent of all policy-makers will read this section, not the whole report".

The report is due to be approved at the IPCC's next plenary session in Rome next month. **Ehsan Masood & Ayala Ochert**

Re-engineering proposed for French agency

Paris. Guy Aubert, director-general of the French Centre National de la Recherche Scientifique (CNRS), last week suggested that the agency's engineering sciences activities might be reorganized to improve their coordination with other disciplines and their ability to meet the needs of industry.

Speaking at a conference marking the twentieth anniversary of CNRS's engineering sciences department (SPI), Aubert proposed the setting up of a body to coordinate engineering across the agency. In particular, this body — known as an 'institute' in CNRS jargon — would oversee the growing number of collaboration projects between SPI and other CNRS laboratories in areas such

as medicine, biology and sociology.

According to Aubert, the aim of the institute would be to reduce duplication and identify areas for cooperation. It would provide companies interested in working with CNRS with a single point of contact.

Aubert also proposed that a "national consortium of engineering sciences" should be set up as a "simple and informal" structure with similar aims to those of the proposed institute, but spanning all public research organizations, including the Atomic Energy Commission and the biomedical research agency INSERM. But he emphasized that both proposals were "only at the stage of intentions". **Declan Butler**

Wellcome sets sequencing project in motion

London. Britain's Wellcome Trust has become the first funding agency on either side of the Atlantic to commit firm support for a plan to produce a full-length 'low-pass' sequence of the complete human genome by the year 2002.

As government funding agencies in the United States and the United Kingdom struggle to find the money they hope to raise for such a project, Wellcome announced last week that it had agreed to provide sufficient financial support to the Sanger Centre, at Hinxton Hall near Cambridge, to sequence one-sixth of the genome.

No precise figure has been given. But in a statement, Wellcome says that the money should be sufficient to enable the Sanger Centre to identify at least 500 million of the 3 billion base-pairs in the genome at an eventual estimated cost of less than £0.10 a base pair, giving a minimum figure of £50 million (US\$80 million).

Wellcome is by far the largest biomedical charity in Britain, with a £200-million annual budget close to that of the Medical Research Council (MRC), and the money will be additional to funds originally committed to setting up the centre — named after Frederick Sanger, who has twice won the Nobel prize for chemistry — jointly with the MRC.

The total plan aims to cover about 95 per cent of the complete genome sequence with an accuracy of more than 99.9 per cent. It was hatched last year by John Sulston, head of the Sanger Centre, and Bob Waterston, of Washington University in St Louis, Missouri, with whom Sulston has worked on the sequencing — now nearing completion — of the genome of the nematode worm *Caenorhabditis elegans*.

After some initial scepticism, a consensus appears to have emerged among molecular biologists that recent mapping achievements — as well as indications that no major advances in sequencing technology can be expected in the near future — suggest that the time has come for an attack on the complete human genome.

Sulston says he is keen that the project should be truly international, with strategy determined by the participating groups rather than by their funding agencies. "This is not a race; we are open to any type of cooperation [with other laboratories] or splitting up of the genome," he says.

Reflecting this approach, Sulston and Waterston felt the optimal division of labour was for one-third of the sequence to be determined by the Sanger Centre, one-third perhaps in Waterston's laboratory, and one-third by other participating laboratories.

Interested US laboratories are now awaiting the result of reviews of more than 20 applications received in response to a request for proposals sent out earlier this year by the National Center for Human

Genome Research (NCHGR) of the National Institutes of Health (NIH).

Final decisions are expected early next year. But there is still some uncertainty about how much money will be available. Although additional funds for mega-sequencing projects have been sought by the Clinton administration in its NIH budget request for the fiscal year which started on 1 October — and have been given priority by Congress during lengthy budget negotiations — final details have yet to be agreed.

NCHGR officials say they are optimistic that the money will be found one way or another. Indeed, they claim that enough progress has been made on mapping projects to make possible the reallocation of sufficient funding within its \$114-million annual budget to meet the sequencing requests, even if no new budget figure is agreed and the NIH remains funded on the basis of a continuing resolution.

In Britain, there is similar uncertainty over the money promised, at least informally, by the MRC. Earlier this year, in releasing the science budget for the financial year

1995–96, the government announced that this would include a special item of £9.6 million for genome research and immunology. But beyond a commitment of £2 million to complete the *C. elegans* work, no announcement has yet been made of what type of long-term commitment the MRC may make to human genome sequencing.

The Wellcome Trust hopes that last week's announcement, which it says will allow the Sanger Centre to "spearhead" the international sequencing programme, will help to catalyse efforts elsewhere, and is keen that the resulting sequence should be in the public domain.

According to Sulston, the precise division of labour will be determined by the interests of the laboratories that eventually agree — and are funded — to participate, but is likely to be based on different institutions taking responsibility for different chromosomes.

Sulston says that Wellcome is planning to organize a meeting next year of the major laboratories involved in the area to discuss questions of strategy. "We want to share the leadership," he says.

David Dickson

Genetic testing 'needs more checks'

San Francisco. US academic and commercial laboratories are rushing to offer genetic testing with a minimum of external review, according to a new survey that reinforces concern among geneticists about the quality of laboratory testing in the United States, as well as the interpretation of results.

The results of the survey led to demands from scientists at the annual meeting of the American Society of Human Geneticists in Minneapolis, Minnesota, last week for a system to evaluate the accuracy and interpretation of tests, as well as turn-around times.

One academic geneticist cited a commercial laboratory that had taken a year to provide test results — and even then had missed two mutations that she had already identified. Others claimed that federal regulations on laboratory quality fail to address the specific technology involved in genetic testing. Furthermore, institutional review boards, which tend to be responsible for evaluating the quality of tests offered as part of a research project, may not have any background in genetics.

Rebecca Anderson, chair of the genetic services committee of the National Society of Genetic Counsellors, said she has great confidence in most laboratories, but would like to see proficiency tests in place. More difficult, she said, may be problems of interpretation of the tests, especially by primary care physicians.

Other geneticists warned of a particular danger in the tendency to use information about high-risk families to make predictions

about the general population. Launching population screening for diseases such as cancer or Alzheimer's without more quantitative data about the prevalence of certain mutations would produce only "garbage" information. "It doesn't mean we stop. We just have to be very careful," said Michael Watson, a medical geneticist at Washington University School of Medicine in St Louis, Missouri, and head of the laboratory practices committee for the American College of Medical Genetics.

Another specific concern is the use of genetic tests on children. In general, geneticists have agreed not to carry out such tests on minors unless they are likely to benefit directly. But a group of researchers led by Dorothy Wertz at the Shriver Center in Massachusetts surveyed 186 laboratories listed in Helix, a national directory of DNA diagnostic laboratories, and found a high proportion that tested healthy children.

Results of a survey of academic and commercial laboratories that are developing or offering genetic tests on an experimental basis were presented by Neil Holtzman of Johns Hopkins Medical Institutions in Baltimore, Maryland. The study is intended to inform the work of the Task Force on Genetic Testing for the Human Genome Project, which is developing guidelines for the scientific validation of genetic tests, the assurance of laboratory quality and general education and counselling.

Out of 54 biotechnology companies in the survey that said they were developing genet-

ic tests, 41 per cent had never contacted either the Food and Drug Administration (FDA) or their own institutional review board (IRB). Similarly, out of 140 academic laboratories, more than a quarter had never contacted either organization.

Holtzman pointed out that organizations that provide services and make their own probes and primers must ask for review from their IRB, and in some cases must also obtain an investigational device exemption from the FDA. External review did increase among the 14 biotechnology companies offering services using their own products. But almost 30 per cent still had never contacted a regulatory body.

The study also showed that academic laboratories were less likely than companies to have undergone any external quality review. Out of about a hundred non-profit laboratories providing molecular genetic services, 16 had not registered as required under the federal Clinical Laboratory Improvement Amendments or participated in any proficiency testing. At least one geneticist said he felt his published discoveries were sufficient to demonstrate the quality of his laboratory, and that certification was unnecessary, says Holtzman.

Some participants at the Minneapolis meeting claimed that the survey was too imprecise to demonstrate the size of the problems described. They criticized the results for not indicating how far along the development process a company had progressed before approaching any regulatory body. Stephen Goodman, of the University of Colorado Health Sciences Center in Denver and treasurer of the American Society of Human Genetics, said that a laboratory was not necessarily in the wrong if it had not approached its institutional review boards. "You really need to know the research they're doing," he said. **Sally Lehrman**

NASA staff face shake-up as panel backs privatization bid

Washington. The concept of privately run "science institutes" has been endorsed in an internal study at the US Aeronautics and Space Administration (NASA). The institutes would conduct a significant amount of the agency's research and — presumably — inherit many of its staff scientists.

But the plan is still vague, and fraught with legal and administrative problems. Scientists inside and outside the agency are reserving judgement until they hear more about it. "We're all still waiting to see the details," says Anneila Sargent of the California Institute of Technology, chair of NASA's Space Science Advisory Council, which will be briefed on the plan next week.

The idea of science institutes surfaced last winter during a series of soul-searching reviews intended to lead to a new, leaner space agency (see *Nature* 374, 107; 1995).

Initially, 'privatizing' NASA's science was seen as a way to cut the agency's operational overheads. But by the time Daniel Goldin, the NASA administrator, announced a plan to restructure the agency in May, the institutes were no longer being touted as money-savers but as a way to enhance the quality of NASA research by strengthening its ties with industry and the academic world.

A team led by Al Diaz, the agency's deputy science chief, spent much of the summer touring NASA and non-NASA centres, formulating a plan of the way in which the institutes would be structured. Diaz is now beginning to go public with his group's findings, briefing officials in Congress and the White House, as well as NASA advisory

groups, on the plan's broad outlines.

The new institutes, selected through competitive bidding, would receive their core funding from NASA; existing NASA centres would provide "services and support" — although what that means has not yet been spelled out. Not surprisingly, the institutes would be encouraged to seek outside funding and become as self-sufficient as possible. To that end, they would retain intellectual property from any research.

The Diaz team began and ended with the same list of 11 institutes proposed by another NASA review team earlier this year. According to Diaz himself, it agreed that a biomedical research institute affiliated with the Johnson Space Center (JSC) in Houston should be the first one established, perhaps by early next year.

The rest are in "various stages of readiness", he says. Three — a Goddard Institute for Space Studies in New York, a Global Hydrology and Climate Center at the Marshall Space Flight Center in Alabama, and an Astromaterials Institute at Johnson — could be created by simply modifying existing relationships.

Three others need to be "further defined", according to the Diaz report. These are institutes for microgravity and space science at Marshall and an Atmospheric Sciences Institute at the Langley Research Center in Virginia. And two more — a space power and propulsion institute at the Lewis Research Center in Cleveland, Ohio, and the National Space Science Data Center, at Maryland's Goddard Space Flight Center — should be reconsidered altogether, because "the model developed for other institutes is not applicable" to them.

The two remaining institutes — for astrobiology (Ames Research Center) and microgravity studies (Lewis) — are also problematic, as Congress might have to pass new laws to clear up thorny employment issues. These include the transfer of pensions and other benefits for government scientists who move to the private sector.

Another issue to be resolved concerns restrictions on where and how ex-government employees can work. NASA officials have already begun talking to congressional committees about passing "legislative relief". Without it, NASA scientists could lose benefits if they move to an institute.

But Diaz points out that the institutes should not be seen as a placement service for redundant government scientists. "The staffing of these institutes ought to be totally at the discretion of the institute management," he says, adding that NASA should not play a role in choosing who gets hired.

Tony Reichhardt

Infrared observatory prepares for lift-off

Munich. The Infrared Space Observatory (ISO) is to be launched from Kourou, French Guiana, by the European Space Agency (ESA) this weekend. The observatory's Ritchey-Chretien-type telescope (right), which has a focal length of 9 metres and an aperture of 0.6 metres diameter, will turn its sights on some of the quarter-of-a-million point sources of infrared radiation catalogued by IRAS, the joint UK/Dutch/US Infrared Astronomical Satellite, which scanned the infrared sky in 1983.

ISO's high sensitivity will give astrophysicists the opportunity to glean fundamental information about the formation of stars and solar systems. ISO will also search for the Universe's 'missing mass' — the difference between the theoretical mass of

IMAGE
UNAVAILABLE
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the Universe deduced from gravitational calculations and what can be seen in the visible light range. □

ESA

Eugenics row inflames vote on faculty name

Paris. The University of Claude Bernard in Lyons, France, is to vote on a controversial motion to remove posthumously from its faculty of medicine the name of Alexis Carrel, winner of the Nobel prize for physiology or medicine in 1912, following new evidence that he may have belonged to France's main fascist party around the Second World War.

Carrel's prominent role in the eugenics movement, in particular in the United States before the war, is no secret. But the winner of the Nobel prize for work on organ transplantation and cell culture is now alleged also to have been a member of the Parti Populaire Français (PPF).

The university council will vote on the issue before the end of the year. But the debate extends far beyond the naming of the faculty. The combination of France's pride in one of its most distinguished scientists, soul-searching over its collaboration with the Nazis, and current concern over extreme right-wing nationalism, makes an explosive cocktail that is dividing historians, political groups and the university itself.

The most recent allegations were made last month by *Golias*, a magazine published by progressive Roman Catholics, which reproduced a 1938 issue of the PPF's weekly newspaper *L'Emancipation nationale* with Carrel's name at the top of a list of intellectuals described as party members. *Golias* and several human rights organizations have issued a joint statement calling for Carrel's name to be removed from the faculty. The magazine and its supporters ask whether it is "acceptable and moral" that students taking the Hippocratic oath should train under the patronage of a man affiliated with a "racist, anti-Semitic and xenophobic" party.

Their call has been "completely supported" by the French socialist party, according to Jean-Vincent Jihanno, a party spokesman. Jihanno says that the socialists on Lyons city council will also seek to have Carrel's name removed from a Lyons street. The extreme right-wing National Front, however, opposes changing the name of the faculty.

Three years ago, the university set up a commission to investigate Carrel's past, following concern about his role in the eugenics movement. Later the same year, a majority of the university council voted to remove Carrel's name from the faculty, but failed to obtain the necessary absolute majority.

Nevertheless, the university subsequently issued a statement denouncing Carrel's eugenic ideas, and added a suffix to the plaque outside the faculty emphasizing that he was being honoured "exclusively" for his Nobel prize and contribution to medicine.

The compromise reflected a sentiment the commission shared with many scientists, namely that patronage honours the scientific work of an individual without necessarily

holding them up as paragons of social behaviour. The commission also noted that renaming the faculty would set a precedent that could require the renaming of streets and buildings named after such illustrious figures as Voltaire, Blaise Pascal or even Louis Pasteur, who in 1884 proposed to the Emperor of Brazil that condemned prisoners be used for testing a rabies vaccine and the effects of cholera bacillus.

The university denies that the decision to give its council another vote on the naming of the faculty has been prompted by the

IMAGE UNAMIGABLE FOR A CRYABGET FOR A SIGHT REASONS

Carrel: a Nazi collaborator, or an apolitical scientist reflecting the views of his time?

latest allegations, arguing that it merely follows a "review" of the issue. But human rights organizations, pointing out that this review has been under way for three years, claim that the university has been trying to "bury the dossier".

These organizations argue that the university should review the commission's 1992 conclusion that, though a *Pétainiste*, Carrel was neither a member of a political party, nor a collaborator. The new evidence, they say, refutes the claim that Carrel was merely a scientist with no political ties.

Carrel — who at 39 was then the youngest-ever recipient of the Nobel prize — received the award shortly after moving from Lyons to the Rockefeller Institute in New York. He later became involved in the US eugenics movement.

In 1935, three years before he returned to France, Carrel published *L'Homme cet inconnu* ("Man, that unknown"), a book that proposed a eugenicist blueprint for replacing democracy with a 'biocracy' and warned that medical progress risked degenerating 'the great white race' by promoting the survival of the weak, and a consequent increase in mental illness and criminality. The book was translated into 19 languages.

According to Philippe Videlier, a historian at the Centre National de la Recherche Scientifique (CNRS) in Lyons, the latest allegations demonstrate a firm link between Carrel's eugenic and racist theses and the policies of the collaborationist regime of Vichy, which handed over 80,000 French Jews to the Nazis. "Carrel was in phase with,

and representative of, Vichy, and vice versa," he claims.

Indeed, Marshal Philippe Pétain, the head of the Vichy regime, met Carrel in 1941, and subsequently provided him with FF40 million to set up the so-called French Foundation for the Study of Human Problems. The university commission concluded that the aim of this foundation — which was dissolved after the liberation of Paris in August 1944, a few months before Carrel's death — was to "prepare a profound reform of French and Western civilization, by creating an élite of researchers to study biological, moral and political problems".

Nonetheless, the significance of Carrel's links with the Vichy government remain hotly contested. Alain Drouard, for example, a CNRS historian at the Centre Roland Mousnier in Paris, and an authority on Carrel, maintains that although Carrel had a political agenda, it was independent of that of the Vichy regime. He also points out that Carrel turned down an offer in 1942 to become secretary of state for health.

Drouard also argues that although Carrel may have attended PPF meetings, the 1938 claim by the PPF's newspaper is not formal proof of Carrel's membership of the party. Carrel belonged to no political party, asserts Drouard, who was a member of the university commission set up to investigate Carrel.

"It's too easy to say Carrel equals Vichy, equals Nazism", says Drouard, claiming that Carrel's views have been taken out of their historical context, in particular the widespread support for eugenic ideas among scientists in the 1930s.

For example, Carrel recommended that criminals — from armed robbers to those who had "seriously abused public confidence" — could be "economically and humanely eliminated" in a "euthanasia establishment equipped with an appropriate gas". Opponents of Carrel argue that this anticipated the Nazi gas chambers. But Drouard claims Carrel was only proposing a widening — "albeit extreme" — of the grounds for capital punishment.

Drouard's general arguments are supported by Gérard Fontaine, the vice-chancellor of the university. Fontaine says that whereas he originally favoured changing the name of the faculty, he has since changed his mind. "It would be too easy to simply concede to public opinion," he says. "A university's job is to reflect in serenity [on the historical facts]."

But human rights organizations argue that the debate is far from academic. They point out that Carrel's theses have been revived in publications of the extreme right wing French National Front party. Maintaining his patronage of the faculty implicitly lends credibility to the policies of the National Front, they argue. **Declan Butler**

Harlingue-Vollet

South African reforms slow to take shape...

Cape Town. South Africa's Minister of Arts, Culture, Science and Technology, Ben Ngubane, took the opportunity of the annual Zuckermann lecture, which he delivered in London last week, to elaborate on his plans for changing the country's science system (see below).

But his speech rang hollow among many scientists within the country itself, who are becoming increasingly exasperated with the apparent lack of progress in putting ideas into practice. "He is a polite recipient of invitations and applause, but has yet to make a direction-setting statement about science and technology," commented one senior research council official.

Unable to resist the English penchant for understatement, Ngubane — who describes himself an optimist — admitted that "there is much to be done" in developing of science and technology in South Africa. But concrete achievements so far seem to have been limited. For example, in his speech Ngubane claimed to have "initiated the process to form a National Advisory Committee on Science and Technology Policy", a body that he announced last February would be in place by June this year.

It remains unclear whether the apparent lack of progress reflects administrative fail-

ures within his ministry, or Ngubane's own preoccupation with developments in his Inkatha Freedom Party. The party suffered further setbacks last week, with the decision of the attorney-general of Natal province to prosecute the party's deputy secretary-general, along with Magnus Malan, South Africa's former Minister of Defence, and ten former military officers, over the massacre of 13 civilians in 1987 by one of its alleged hit-squads.

Since the sacking of his former deputy minister, Winnie Mandela, in March, Ngubane has been deprived of the excuse that his ministry was beset by political problems. But delays continue. For example, having taken nine months to appoint Roger Jardine as his director-general — the senior civil servant post in the ministry — in February, an equivalent period has elapsed without appointments to two deputy director-general positions, one for arts and culture and one for science and technology.

Ministry officials nevertheless claim that considerable movement is taking place. For example, Ngubane used the Zuckermann lecture to commit his ministry to producing by December a green paper on science and technology, on which the scientific community will then be able to comment. A team of

ten scientists — including representatives from the universities, science councils, the private sector and the ministry — had completed three weeks of deliberations on the paper two days previously.

An explicit acknowledgement that South Africa "devotes a large part of its scarce funds for science and technology to costly military and nuclear programmes" is among the terms of reference set for the task force by the ministry. The green paper will serve as a precursor to a white paper, to be tabled during next year's parliamentary session, which will guide the budgeting process for the 1997–98 financial year.

As far as the science budget for the 1996–97 financial year is concerned, the ministry has taken a small step towards reallocating funds between the research councils. Faced with a nominal increase of only 4.6 per cent in the science budget next year, the ministry decided to allocate 90 per cent of the total in the same proportions as it did this year.

But the presidents of the councils were invited to bid for the remaining 10 per cent by making presentations to a panel of civil servants, academics, parliamentarians and trade union officials, chaired by Jardine, on how well their respective programmes complied with the Reconstruction and Development Programme.

This process was completed last month, and recommendations have been submitted to the Ministerial Committee on Science and Technology (MCST), made up of the eight members of the cabinet whose ministries are responsible for administering research funds. This committee, chaired by Ngubane, meets next week, and has the final say in how the allocations are made.

But Ngubane has so far resisted demands to increase the transparency of the process by making the panel's recommendations public. This has left the council presidents free to lobby their ministers, who sit on the MCST, to intervene on their behalf.

Many scientists continue to argue that this system needs to be changed if funding is to go to the most deserving projects, and that increased transparency is an essential step in this direction (see *Nature* 362, 384; 1993). But so far, Ngubane appears to prefer sticking with the old system.

President Nelson Mandela, speaking last month at an international conference on cyclotrons and their applications in Cape Town, made a characteristically impassioned plea to scientists, engineers and academics "to resist the temptation to emigrate" as "the nation needs their collective creativity and ingenuity". So far, however, Mandela's well-intentioned move of setting up a separate ministry appears to many to have done little to secure the long-term viability of South African science.

Michael Cherry

...as minister seeks to rally researchers

London. South African researchers were urged last week by the cabinet minister responsible for science, Ben Ngubane, to support efforts not only to develop high technology but also to help secure the country's internal development.

Delivering the annual Zuckerman lecture in London — named after the late Lord (Solly) Zuckerman, who was himself born in South Africa, and who was the chief scientific adviser to successive British governments in the 1960s — Ngubane said the country's science and technology system had been distorted as a result of the "siege economy" that characterized the last 20 years of apartheid.

One of its current needs was to develop forms of technology that will make South Africa's relatively unskilled professions more self-sufficient. "New subsistence technology" was required in fields such as energy use and infrastructure

development. Innovation and the deployment of talent was just as necessary in these fields as in more conventional areas of high technology, and were neither cheaper nor less complex. Indeed, Ngubane even admitted that working on such technologies creates what he described as "greater career risks for scientists".

Ngubane pointed out that much remained to be done to boost business confidence and accelerate economic growth in South Africa. But, while admitting that South Africa spends a much lower proportion of its resources on research and development than industrialized nations, he said the government's desire to pursue "sound fiscal policies" meant that "changes in our current spending patterns cannot be dramatic".

Ngubane also announced details of a new collaboration fund agreed earlier this year between the British and South African governments, and worth a total of £600,000 (US\$960,000) over a period of three years. The fund will in particular be used to support research projects and fellowships in the fields of agriculture/biotechnology, biomedicine and the environmental protection and use of natural resources.

D.D.



Ngubane: science must help in reconstruction.

Causes of death in haemophilia

SIR — Darby *et al.*¹ convincingly demonstrate the impact of HIV infection on mortality among haemophiliacs. Their study raises another important issue, the high mortality rate from bleeding and liver disease among people with haemophilia, both HIV-infected and un-

tors to mortality in our cohort. It is interesting that very similar data were obtained two decades earlier, before the AIDS era.

These studies show why we were then so concerned with bleeding in our patients, and why we should be just as concerned with bleeding in haemophiliacs

Study:	CAUSES OF DEATH					
	HIV+		HIV-			
Total no. of deaths by HIV status	Darby ¹ 403	MHCS ² 389	Darby 84	MHS 22	Aledort ³ 206	Forbes ⁴ 62
Year of study	1995	1995	1995	1995	1977	1977
Cause of death*						
AIDS** ± other	235(58.3)	280(71.9)	—	—	—	—
Bleeding	73(18.1)	27(6.9)	38(45.2)	7(31.8)	85(41.2)	19(30.6)
Liver disease	11(2.7)	30(7.7)	8(9.5)	3(13.6)	9(4.3)	5(8.0)

* Number of (per cent) of HIV (+) or (−) deaths

** AIDS indicates opportunistic diseases that meet the 1987 surveillance definition of the US Centers for Disease Control and Prevention

infected. They showed a 307-fold and 1,156-fold increase in bleeding deaths for HIV-infected and -uninfected subjects, respectively, when compared to the general population. Similarly, deaths from liver disease were increased 37-fold in the HIV-infected subjects and 16-fold in the uninfected subjects.

We analysed data on mortality from the Multicenter Hemophilia Cohort Study (MHCS) to determine if bleeding and liver disease were also frequent causes of death for our patients. Our cohort is well described², and includes subjects from haemophilia treatment centres in the United States, Greece, Germany, Austria, Switzerland and France. Mortality data are collected at the time of death, rather than from death certificates, and in our study multiple causes of death can be listed for each decedent. For this analysis, death from bleeding included subjects who died from only bleeding without other causes of death listed. Deaths from liver disease included some patients dying of liver disease and other illnesses, but not AIDS-associated opportunistic diseases. Data were also obtained from two early studies of haemophiliac mortality, one from US and European centres³, and another from haemophilia treatment centres in the United Kingdom⁴.

As the table shows, our current data confirm the findings of Darby *et al.* Bleeding and liver disease are major contribu-

now. Clotting factor concentrates and blood products must be used more effectively to treat and prevent the bleeding that continues to threaten the survival of these individuals.

W. Christopher Ehmann

M. Elaine Eyster

Department of Medicine,
Division of Hematology,
Pennsylvania State
College of Medicine,
Hershey,

Pennsylvania 17033, USA

Louis M. Aledort

Department of Medicine,
Mount Sinai School
of Medicine,

New York,

New York 10029-6574, USA

James J. Goedert

Viral Epidemiology Branch,
National Cancer Institute,
Rockville, Maryland 20892, USA

Nicotine as a drug

SIR — I am writing in response to your leading article "Nicotine as a drug" (*Nature* 376, 539; 1995). Most governments are in a dilemma about the contentious issue of saving people from tobacco addiction and related afflictions. The enormous revenue and the employment potential in tobacco cultivation, processing and marketing acts as a temptation not to ban tobacco use. Most governments do not go beyond insisting on a statutory warning about the health hazard on cigarette packets (often printed illegibly). The knowledge that tobacco generates jobs for doctors and other health-care workers is not recent.

In India, tobacco smoking is primarily a

male vice. One of the strategies used in advertisements aimed at young males encouraging tobacco addiction is projecting tobacco (cigarette) smoking as manly, and something that women like. Women should make explicit their dislike for the use tobacco. Advertisements to this effect in all media would be very helpful. This is true also in other countries where tobacco smoking is largely restricted to men. Feminist organizations must help to create a 'smokeless world', with the same zeal as they fight for equality in rights and status.

It is also necessary to find better uses for the land traditionally used for tobacco cultivation. In India at least, strawberry and sugar-cane cultivation would offer effective alternatives with the same employment potential.

S. John

Shibu Nivas, Sagar Park,
S. No. 46/9, Nagar Road,
Pune 411014 India

Tonegawa defended

SIR — As members of Dr Susumu Tonegawa's laboratory, we should like to comment on the news report "Nuclear regulators to investigate 'contamination' incident at MIT" (*Nature* 377, 563; 1995). The report contains inappropriate speculation and factual errors, exemplified by the incorrect account of the work for which Tonegawa was awarded the Nobel prize.

We are greatly disturbed by the prejudicial tone of the report. In its context, the statement that Tonegawa is reputed to bring "an intensively competitive approach to his laboratory" implies that he fosters competition within his group and is therefore indirectly culpable for the unfortunate incident that has occurred. We should like to state emphatically that Dr Tonegawa does not encourage competition within his group.

**A. Abielovich, C. Chen, D.F. Chen,
J. Delaney, D. Gerber, L. Graziadei,
M. Hasan, H. Hinds, A.A. Hsu, J. Huang,
S.Y. Huang, P. Huerta, T. Iwasato,
D. King, P. Künzler, J. Lafaille, C. Levelt,
D. Lim, C. Lovett, S. Marusic-Gaselic,
T. McHugh, K. Nakazawa, H. Prosser,
Z. Qian, T. Sasaoka, J. Shen,
F. Van de Keere, Y. Wang, M. Wu,
M. Xu, R. Zacks**

Center for Cancer Research,
Massachusetts Institute of Technology,
Building E17-383,
77 Massachusetts Avenue,
Cambridge,
Massachusetts 02139-4307

■ The error about Dr Tonegawa's Nobel prize was introduced in the editing — Editor, *Nature*. □

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Benefits of placebos

SIR — The gold standard for demonstrating efficacy in modern therapeutics is the placebo-controlled, double-blind (masked) clinical trial. Beatrice Golumb¹ makes the point that it is difficult to exclude small systematic effects caused by the placebo, particularly when used over a long period. In order to try to counteract this possibility, it is the usual practice of pharmaceutical companies to employ the same formulation in both the placebo and the active treatments. Ideally, the only difference is the omission of the putative active substance. Details of both active and placebo formulations are conventionally provided to regulatory authorities in clinical trial and drug registration applications. They are rarely, if ever, published in the literature. The precaution of using similar formulations does not negate Golumb's argument. However, it is difficult to construct a test to demonstrate the inertness of a placebo. The proof involves the difficult concept of showing that the placebo never has any action, that is, proving a negative finding. Furthermore, the demonstration would itself require a placebo comparison, a situation reminiscent of the rhyme: "Dogs have fleas upon their backs to bite 'em, little fleas have lesser fleas, and so *ad infinitum*".

Schoemaker², in commenting on the problems of placebo, raises several additional aspects including the perception that regulatory authorities are driving an unnecessary and inappropriate use of placebo in clinical trials³. A so-called 'pivotal' study does not necessarily involve a placebo control. It is merely a term arbitrarily used to describe the biggest, best and most convincing studies conducted to support a registration application. There are obvious areas in which the use of a placebo is unethical — infectious disease, for example. More difficult areas are diseases characterized by relapse/remission cycles, for example endogenous depression. In this situation, changes in the activity of the disease will occur, with time, in the absence of any treatment. Thus an improvement in an 'active' treatment group may be partly due to the new treatment and partly to natural remission. Without a placebo comparator, the magnitude of the two effects cannot be resolved. Any improvement in a placebo-treated group, studied in parallel, allows an assessment of the relative contribution of the two effects.

Obviously, the benefit of using a placebo, in terms of improved power to predict the efficacy of drugs must be balanced against the hazards of denying patients beneficial treatment. Evidence of such denial has recently been published in studies of postoperative nausea and vomiting⁴, and it is probable that it also occurs

in other therapeutic areas. In order to combine stringent evaluation of pharmaceutical products and correct management of patients, it would appear desirable that a transition phase should exist in the life of a drug under development, at which superiority over placebo is considered to have been demonstrated. That demonstration should act as a trigger to switch the process of evaluation to comparative studies with existing treatments. How such a switch is to be effected, whether before or after licensing, who pays for the studies, who monitors the results — all these are legitimate questions that have hardly been posed, and the answers are a long way over the horizon.

The opinions expressed above are personal, and are not necessarily shared by the board that I serve.

David Lyons

National Drugs Advisory Board,
63 Adelaide Road,
Dublin 6,
Ireland

SIR — According to *Webster's Dictionary*, placebo (Latin; *I shall please*) is a (usually pharmacologically inactive) "medicine given merely to humour the patient" without regard to disease. In this respect, placebos can become powerful tools in expert hands to relieve patients from misery and are a substantial therapeutic approach in primitive medicine. Problems may arise¹ when putative therapeutic agents are tested under the misconception that placebo control is a zero-reference condition only reflecting changes related to the experimental setting.

By contrast, in otherwise controlled experiments, placebos may be as dependable a reference as the third dimension of holograms. This may particularly apply to normal and diseased brain functions, which reflect interactions of metabolic or hormonal factors and neural machinery: the action of steroids on CNS, the glucose-dependent, ATP-regulated K channels linking neuron excitability and metabolic state⁵, and the therapeutic effect of antidepressants through action on the hypothalamic-pituitary-adrenocortical system⁶ are examples. It is true that, in these circumstances, the effect of spontaneous changes in (drug-unrelated) factors may interfere with or even mimic drug action. Biased results are a possibility in placebo-controlled trials no matter how accurate they are, and a direct control of all variables is neither realistic nor cost-effective. It is also true, however, that biased results are potentially instrumental in generating working hypotheses for further research and that progress in physiology and pharmacology should help to identify potential-

ly critical, drug- or treatment-unrelated factors. Some degree of control of spontaneous variability should be possible.

The alternative to placebos (comparison with known effective drugs) may not serve its purpose either. The development of new antiepileptic drugs (once serendipitous and now based on expanding scientific opportunities but far greater effort)⁷ is an example. Putative antiepileptics are first screened in clinical trials as add-on treatment for otherwise drug-resistant patients. This means test compounds are administered in association with established (standard) drugs that have already proved ineffective in any single patient and which it is hoped may prove more efficient. Ethical restraints on discontinuing treatment⁸ and the confounding effect of drug withdrawal motivate this approach. A modest therapeutic potency is the best expected result in these conditions, whereas adverse effects are often frequent and of ambiguous attribution.

The chance is therefore small that new antiepileptic drugs that are effective and safe for the majority of patients (who luckily are not drug-resistant) will be identified, in spite of effort, investment and labour. There are ethical counter-arguments on placebo use⁸ yet both 'gold' standards and placebos as we know them today may be inadequate control conditions. This is not so trivial a problem in clinical pharmacology as it may seem — and it is one that has long been neglected.

Walter G. Sannita

International Pharma-EEG Group,
University Hospital San Martino,
16132 Genova,
Italy

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Novel satellite

SIR — I admire Nersi Razavi's audacious plan to promote cultural tolerance by means of an orbiting double balloon, reported by Steve Nadis (*Nature* **377**, 281; 1995). This profound experiment is clearly the first step to a new science of astrological engineering. But before funds are committed to the venture, its proposers should clarify two crucial details: (1) the mechanism by which the novel satellite will augment cultural tolerance and (2) the chance of it working.

David Jones

Department of Chemistry,
University of Newcastle,
Newcastle upon Tyne NE1 7RU, UK

Spanish science reaches a crossroad

Pere Puigdomènech

The prospect of a change of government in Spain is unsettling the country's scientific community. But the growth in scientific activity over the past 13 years of socialist rule is also now showing signs of exhaustion.

BRIEFLY in eighteenth-century Spain during the reign of King Carlos III, while California was being populated, Spanish science began to follow the European trend. The Royal Botanical Garden opened in Madrid, and groups of scientists flourished throughout Spain and collaborated with those in other countries. But this growth did not survive the change in royal interest, and during the nineteenth century Spanish science fell into decline. Spanish scientists are now worried that history may be repeating itself.

Spain is on the brink of an important political change that could bring into power the conservative Popular Party or a new type of coalition government. This would mean the end of the socialist government led by Felipe González that has ruled since 1982. For science it means the end of a period that has seen an important increase in the quantity and quality of research. Spanish science faces either a socialist programme that has been showing signs of exhaustion during the past five years or a conservative programme that is an unknown entity. Either way, Spanish scientists are worried that radical changes or lack of priority in the scientific system will harm a fragile and young science that still needs consolidation.

During the past 12 years, funding for Spanish science has dramatically increased. From 1980 to 1990, the public spending for research more than doubled (even after correction for inflation), as did the number of positions in universities, and the research staff in CSIC (Consejo Superior de Investigaciones Científicas), the main public research institution, increased by 50 per cent. In the same period, the public system of research and higher education was considerably reorganized. Parliament approved the Law for University Reform (Ley de Reforma Universitaria), which produced greater autonomy for universities, and the Law for Coordination of Research (Ley de la Ciencia), which defined the organization of public science. In particular, the National Plan for Research (Plan Nacional) was designed to coordinate different departments and to orientate research, and the National Agency for Evaluation was established to evaluate all the grants funded by public agencies through a peer review system. The evaluation system has gained a reputation of independence and credibility.

The number of publications in inter-

national journals has grown by 9 per cent a year and the relative impact of publications by Spanish scientists has doubled in the past ten years. Some areas such as clinical sciences, molecular biology, material sciences, theoretical physics and chemistry are cited among the most productive.

But the socialist government carried out most of its policies between 1982 and 1990. Since then, the trend has taken a downward turn. Between 1990 and 1995, four ministers passed through the Ministry of Education and Science, and the budget for research levelled off or even decreased slightly. As a result, the spending for research in relation to gross national product has not even reached the goal proposed for 1991 of 1 per cent (the average spending in the European Union is 1.9 per cent), and the number of scientists per 10,000 inhabitants (26) is still half the average for European Union countries.

At the same time, some of the weaknesses of the system have become apparent. Take personnel. In 1994 there were more than 5,000 graduate students in Spanish laboratories. They make up the main workforce in research groups and are encouraged to train abroad after gaining their PhD. It is difficult to know exactly how many Spanish postdocs are currently working abroad, but Spain has more applicants for postdoctoral European fellowships than any other country. This inevitably gives rise to several problems: only 28 per cent of postdocs work in private companies; public research institutions are only just managing to replace staff going into retirement; and 95 per cent of new positions in universities are filled by departmental candidates. Lack of mobility and permeability between institutions is a handicap for young scientists. A programme of contracts for postdocs has attracted young scientists, yet many of them find out at the end of their three-year appointment that no further opportunities are offered to them.

The situation is particularly bad in CSIC where around 400 postdocs are under contract but only 50 positions are offered this year. This may be due to the hesitations about CSIC itself. Although universities have become directly involved in research, the size of the budget and staff of CSIC, which now represents around 20 per cent of Spanish research activity, has been frozen since 1990. The relationship of CSIC with the universities and with the

autonomous governments (more than half of its budget is still spent in Madrid), the structure of its staff (members have the status of civil servants) and its internal organization (scientific discussion is almost nonexistent), are all areas fraught with questions that still need solutions.

Another emerging question is the participation in Spanish science of the autonomous governments which are now in place in all regions. With the involvement of the central government levelling off since 1990, they have become increasingly important. Control of universities has been transferred to them and in some cases the regional investment in science is comparable to or even higher than that of central government. Nevertheless, a way has to be found to allow different levels of administration to cooperate instead of competing. In some aspects, such as the provision of equipment or the establishment of new institutes, this collaboration, as well as European regional funds, could be decisive: space for laboratories is badly needed in the most active institutes and existing funds cannot meet these needs.

The political uncertainty during the past three years has led to an inability to make decisions. The exception may be the Plan Nacional, which has carried out a large consultation for its new four-year scheme (1995–99), although how it is going to be funded remains unclear. Although the socialists are clearly not interested in addressing the questions that remain open, alternative proposals by the opposition Popular Party have not been encouraging. Although it has asserted that research would continue to be a priority under its government, some of the proposals in its programme for the 1993 elections produced commotion in the scientific community. They included replacing the grant and evaluation system with the simple redistribution of funds among universities and research institutions. Additional funds would be left solely to contracts with industry. The Popular Party seems to have belatedly realized that this type of proposal would be unworkable and has started a series of consultations with scientists and industrialists. The road ahead is a long one. □

Pere Puigdomènech is in the Departament de Genètica Molecular, Consejo Superior de Investigaciones Científicas, 18 Jordi Girona, Barcelona 08034, Spain.

How trinucleotide repeats may function

The hereditary neurodegenerative diseases marked by repetitive genetic elements may arise from interaction between the mutated proteins and others specific to the brain.

FIVE hereditary neurodegenerative diseases are now known to involve expansions of the trinucleotide CAG within the susceptibility genes, resulting in an abnormally long stretch of polyglutamine within the encoded proteins. Each of these diseases involves a progressive and often fatal loss of specific populations of neurons; the best-known is Huntington's disease (HD), while the others include spinocerebellar ataxia type 1 (SCA1), Machado-Joseph disease (SCA3), spinobulbar muscular atrophy (SBMA) and dentatorubral and pallidolysian atrophy (DRPLA). Why do expanded stretches of polyglutamine appear to be so damaging?

Two important contributions to answering this question are to be published in *Nature* on 23 November. As chance will have it, the authors will also be presenting an account of their findings next Monday (13 November) at the annual meeting of the Society of Neuroscience at San Diego, California. For the benefit of those who will not be there, or who may read of these important developments elsewhere, what follows is a brief summary of the findings.

A distinctive feature of all these diseases is a phenomenon known as genetic anticipation, whereby the symptoms appear at earlier ages and with greater severity in successive generations. It is now clear that anticipation is due to the instability of the amplified DNA sequence; as it passes through the germline (particularly the paternal line, which undergoes more cell divisions per generation than the maternal line), the number of repeats increases. A recent study suggests that the repeats form hairpin structures that disrupt normal DNA replication (*Cell* **81**, 533–540; 1995); once the number of uninterrupted repeats exceeds a critical threshold, it may form a stable hairpin, thereby leading to a vicious circle of replication errors and further expansion.

To explain the amplification of trinucleotide repeats is one thing, but to understand — and ultimately to prevent — the consequences is another matter. It now appears that polyglutamine expansion renders the altered proteins not merely useless but actively harmful (in genetic parlance, a gain-of-function mutation); each of the five syndromes is genetically dominant, and in cases where the loss-of-function phenotype is known, it does not resemble the disease pathology; loss of huntingtin, the HD gene product, causes embryonic lethality (*Cell* **81**, 811–823; 1995; *Science* **269**, 407–410; 1995) while the SBMA gene encodes the androgen receptor, whose absence leads not to

neurodegeneration but to testicular feminization. Moreover, a transgenic mouse with an expanded SCA-1 gene develops Purkinje cell degeneration, whereas the normal gene has no effect (*Cell* **82**, 937–948; 1995).

If expanded glutamine repeats kill neurons, how do they do it? Polyglutamine occurs in several transcription factors, but an effect on transcription seems unlikely, given that at least some of the mutant proteins are cytoplasmic rather than nuclear (*Nature Genet.* **10**, 3–4; 1995). Another possibility is that polyglutamine may lead to the formation of insoluble aggregates; but there is little evidence that any of the proteins accumulate to unusually high levels in affected tissues. Many suspect that the expansion of the polyglutamine may lead to new and pathological interactions with a target protein, hitherto unidentified.

Now, Chris Ross and his colleagues at Johns Hopkins University School of Medicine have identified a protein they term HAP-1 (for huntingtin-associated protein 1), which binds to the mutant form of huntingtin both in a yeast two-hybrid screen and in transfected cells. It is not yet known whether it also binds to huntingtin in the brains of affected individuals — a crucial question — but the restriction of its expression to the brain could explain why the mutated huntingtin protein kills brain cells while leaving other tissues unscathed. Most important, the strength of binding between HAP-1 and huntingtin depends on the number of glutamines; wild-type huntingtin, with 19–22 repeats, binds only weakly, whereas the mutant forms bind more strongly as the length of the polyglutamine stretch increases from 44 to 82 repeats. Length is probably not the only factor, because atrophin-1 (the DRPLA gene product) contains 21 glutamines but shows no detectable binding. Whether longer, pathological forms of atrophin-1 (*Nature Genet.* **6**, 9–13; 1994) can bind HAP-1 remains unknown.

The case for HAP-1 involvement in HD is still circumstantial, and will remain so until there is hard genetic evidence. Optimists may hope that a HAP-1 knockout mouse will succumb to progressive dementia and loss of neurons, but it seems equally possible that HAP-1 is activated, rather than inhibited, by polyglutamines. The sequence of HAP-1 offers no clue to its function, although it has at least two relatives that may also prove to be important. In short, the questions are clear but many months of hard work will be needed to answer them.

The second new study, by Jean-Louis Mandel and colleagues (INSERM, Stras-

bourg), strongly suggests that all the neurodegenerative diseases arise from a common mechanism involving a change in protein conformation. The authors have obtained a monoclonal antibody (1C2), which binds polyglutamine stretches both in huntingtin and in the SCA1 and SCA3 gene-products. Like the association between HAP-1 and huntingtin, the strength of 1C2 binding is correlated with the number of glutamine repeats, being largely specific to the mutant forms of these proteins. This may be due to the formation of a so-called 'polar zipper' structure, in which β -strands interact via hydrogen bonding (*Proc. natn. Acad. Sci. U.S.A.* **92**, 6509–6513; 1995). Perhaps when it reaches a certain length, the polypeptide strand can fold back on itself to form a stable hairpin; crystal structures will be awaited with interest.

The two new studies have several immediate implications. First, they offer the prospect of a biochemical assay for the pathogenetic process. It should be simple enough to screen for small molecules that block the interaction, and the fact that glutamine repeats are not highly conserved in evolution and may therefore have no essential function is encouraging, as is the likelihood that the HAP-1 interaction involves an abnormal structural conformation; it should in principle be possible to block it without disrupting any normal physiological process.

The 1C2 antibody also offers a strategy for the identification of further trinucleotide disease genes, and indeed two other diseases (SCA2 and autosomal dominant cerebellar ataxia type II), as yet uncharacterized at the molecular level, also involve the appearance of proteins that react with 1C2 and so presumably contain expanded polyglutamine repeats. Seven hereditary ataxias have been described, and as they all show genetic anticipation, it seems likely that all will involve the same mechanism. Because their pathological manifestations are poorly defined and change with each generation, a biochemical method of diagnosis will be invaluable for isolating the genes involved. But a bigger prize may lie ahead. Bipolar disorder (manic depression) and schizophrenia, which are much more common than the hereditary ataxias, both show genetic anticipation (*Trends Neurosci.* **16**, 254–260; 1993), although attempts to map susceptibility loci have been inconclusive. Neither condition is known to involve neurodegeneration, but this may occur in subpopulations of neurons that have yet to be recognized. A positive result would indeed be a spectacular payoff. **Charles Jennings**

Charting the circuits

Monte S. Buchsbaum

AT what longitude and latitude of the brain's hemispheres does the schizophrenic lesion lie? Two papers in this issue, one by Dolan *et al.*¹ on page 180 and the other by Silbersweig *et al.*² on page 176, describe the application of the powerful surveying technology of positron emission tomography (PET) to image the regional flow of blood, an index of brain activation, to mapping abnormalities in the brains of schizophrenics. Taken together, their results pinpoint limbic structures, such as the cingulate gyrus, as providing important routes of information between the frontal lobe and other brain regions implicated in schizophrenia.

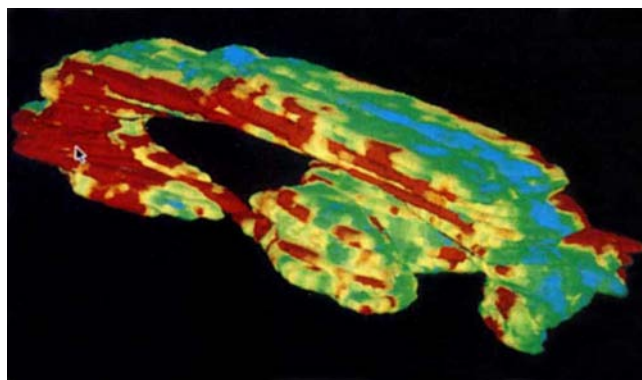
This severe psychiatric illness is typically characterized by symptoms of disordered thought, auditory hallucinations, social withdrawal and attentional deficit. It is considered to have a genetic contribution and is ameliorated by medication that blocks the dopamine receptors. Trying to fit these behavioural and neurochemical clues together to triangulate the location of one brain area or neural system has proved difficult, depending as it must on inferences drawn from brain-injured patients or from the level of neurotransmitter metabolites in schizophrenics' blood.

The past decade has seen the burgeoning of brain-imaging studies of schizophrenia, which have highlighted the frontal lobe, an important site of attention and emotion; the temporal lobe, a centre of speech and perception; and the basal ganglia, central nuclei rich in the neurotransmitter dopamine.

Dolan *et al.*¹ base their investigations on a neurochemical interpretation of schizophrenia that posits it to be a hyperdopaminergic disorder. They treated schizophrenic patients and normal individuals with apomorphine, a dopamine agonist, and acquired images while the subjects were carrying out a verbal fluency task (naming words that begin with a particular letter), a test in which neurological patients with frontal lobe injury often perform poorly.

Patients showed a relative failure of flow increase with the task, but a significantly larger than normal activation following dopamine stimulation in the anterior cingulate gyrus. This finding suggests that the dopamine system in schizo-

phrenic patients is abnormally sensitive, but in areas outside the regions most densely populated with dopamine receptors in the striatum. Direct imaging of dopamine-receptor density in the striatum has generated contradictory results, suggesting that levels are either elevated³ or normal⁴, but these methods have greater difficulty in assessing dopamine function in areas of low receptor density such as the cingulate gyrus.



The cingulate gyrus and thalamus may be important functional elements in circuit deficits in schizophrenia. Here, the outlines have been traced on serial magnetic resonance imaging (MRI) sections of a normal subject, and the MRI outlines coregistered to glucose metabolic images from positron emission tomography (PET). The surface is shown as metabolic rate on the rainbow colour scale (red and orange the most active; green and blue less active). The viewpoint on the brain is that of standing to the left and slightly behind the patient. The anterior cingulate is active and is seen to arch over two globular halves of the thalamus in the brain's midline to become less active in its posterior extent (right). (Image courtesy of Neuroscience PET Laboratory, Mount Sinai School of Medicine.)

On the other hand, the imaging studies of Silbersweig *et al.*² explore patients' hallucinations, the insignia symptom of schizophrenia. Using an ingenious strategy, they asked hallucinating patients to signal by pressing a button each time they heard a voice. More than twenty blood-flow scans were obtained for each patient, during about half of which they were not hallucinating. The authors then statistically analysed the brain for areas in which blood flow increased with the manifestation of the psychotic symptom. The cingulate gyrus, limbic and paralimbic structures, including the hippocampus, the subcortical areas of the thalamus and the dopamine-rich putamen, were all identified.

Both papers chart their statistical explorations with the latitude and longitude of Talairach⁵, who is perhaps the Mercator of brain map projections. Examining, for example, the transverse image shown in Fig. 2b of the paper by Dolan *et al.*¹, we see the intensified apomorphine-induced flow increase centred at the anterior cingulate (coordinates in

millimetres: $x = 8$, $y = 18$ and $z = 28$). Table 1 of Silbersweig *et al.*² shows the anterior cingulate statistical maximum to be close by at 12, 10 and 32 mm, respectively — about 9–10 mm apart as the figurative crow would fly.

Hallucinations are symptoms that are especially sensitive to neuroleptic administration. It is of interest that the hallucination-induced flow increases observed by Silbersweig *et al.* are also reported for the putamen, a structure that shows changes in metabolic rate as a result of neuroleptic dopamine blockade and, in some studies, increased dopamine-receptor density in schizophrenic patients. In contrast, Dolan *et al.* did not observe any striatal change with apomorphine administration in patients doing a verbal fluency task. Thus, mental activity of the patient at the time of imaging is an important determinant of the set of neural structures activated.

Surveying the full group of communicating regions important to schizophrenia would appear to require behavioural states and response to pharmacological probes to be monitored in conjunction with one another. The neuroscience can be no better than the sophistication of the behavioural task probes and the neuropharmacological challenges that researchers employ. Attempts merely to hold the patient down in the scanner may be sufficient for purely anatomical studies with computed tomography or magnetic resonance imaging (MRI), but they cannot reveal the functional circuitry of the largely uncharted domains of psychosis. These studies advance progress towards filling the still sizeable blank areas on the brain map of human psychological function and dysfunction.

In the cartography of schizophrenia, the two atlas-based studies presented here are identifying large structures — continents, as it were — and showing the relationships between them. The next step in the exploration of schizophrenia through PET will more fully exploit the precise delineation of brain structures made possible by MRI. By combining the two techniques (see figure illustrating the cingulate gyrus and thalamus), more

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2. Silbersweig, D. A. *et al.* *Nature* **378**, 176–179 (1995).
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finely grained analyses will focus on the activity within functional subregions and adjust for individual differences in human anatomy. Human and animal data indicate that memory, executive function and vocalization may be related to different specific cingulate subregions⁶ — separating them will prove important in

integrating functional imaging studies within the larger field of neuroscience and in extending the advances made here. □

Monte S. Buchsbaum is in the Department of Psychiatry, Mount Sinai School of Medicine, Box 1505, One Gustave L. Levy Place, New York, New York 10029-6574, USA.

ENTOMOLOGY

Headless hosts, legless guests

Donald H. Feener Jr

DECEPTION and exploitation are common themes in the natural world. As in human society, a more sophisticated 'mark' requires a more elaborate confidence trick. On page 137 of this issue¹, Weissflog *et al.* report the discovery of such a con involving a newly described genus of fly that lives in the colony of a driver ant in the Old World genus *Aenictus*.

Adult females of the fly are extremely aberrant in form and more closely resemble the larvae of their host than a typical adult fly. Structural modifications include the loss of legs and wings, reduction of thoracic and abdominal sclerites, and the elongation of the abdomen into a worm-like shape. Presumably, these extreme alterations in form enable female flies to mimic the complex nest-mate recognition cues of their host, allowing them to live unmolested in the colony and perhaps even to solicit food successfully from workers tending ant larvae.

The discovery by Weissflog *et al.* will quite rightly draw attention to the Phoridae, the family to which the new fly belongs. The Phoridae is little known outside entomological circles, yet its members display a range of habits and body forms that is unrivalled by any other family of Diptera, and possibly by any other family of insects². Members of the family include pests of cultivated mushrooms, parasitoids of such unusual hosts as earthworms and snails, and the macabre 'coffin fly', which successfully reproduces on corpses in coffins². Phorids favour dark, secretive, damp and mouldy places, which preadapts them for life in the colonies of social insects (ants, bees, wasps and termites). Approximately half of the 240-odd genera have formed associations with social insects, and among these about half are exclusively associated with ants².

Ant-associated phorids exhibit two radically different lifestyles^{2,3}. Nest symbionts, such as the fly described by Weissflog *et al.*, spend part or all of their life in the colony of their hosts obtaining food as scavengers in refuse piles, as predators of host ants, their brood or other guests, or by soliciting liquid food from attending worker ants^{2,4,5}. Over 80 per cent of the species with this lifestyle

are associated with New World army ants. A single colony of army ants in Costa Rica or Peru may house more than half-a-dozen species of fly. Adult females of many species are wingless or short winged, and often possess other unusual structural modifications as well.

These variations in body form are correlated with equally diverse behavioural interactions between symbionts and hosts. Some species are apparently fully integrated into the social life of their host,

evolutionary convergence in insects.

The second group of phorids associated with ants are aerial parasitoids of adult workers. Females of these species are freelifing and seek out ant colonies only to lay their eggs in workers. Larval development occurs in the ant's head capsule which eventually falls off, hence the vivid common name 'ant-decapitating flies'. The flies attack a wide assortment of ants including many pest species and competitive dominants². Host ants have responded to the successful radiation of ant-decapitating flies by evolving specialized behaviours for defending themselves against ovipositing females, hitchhikers in leaf-cutting ants being a particularly spectacular example⁶ (see picture). Activation of these defence behaviours often interferes with the ability of workers to harvest food sources or guard them against competing species⁷⁻¹². So the mere presence of parasitoids may be enough to alter the balance between competing ant species or the ability of a particular species to dominate local ecological interactions⁷.

What is remarkable about these systems is that the community-level consequences of the parasitoids arise indirectly from the threat of parasitism rather than from its actual rate. This allows the influence of the flies to be magnified far beyond their abundance, meaning that they may play an under-appreciated role as 'keystone' species in many ant communities. This new perspective on possible regulatory forces within ant communities is now being applied to the control of the red imported fire ant, a species that is devastating arthropod biodiversity in the southeastern United States^{11,12}.

The unusual fly discovered by Weissflog *et al.* is just one of hundreds of ant-associated phorids awaiting description and study. These flies promise to tell us much about the evolution of species interactions and its ecological consequences. And they serve to remind us how little we know about the diversity of the Earth's biota. □

Donald H. Feener Jr is in the Department of Biology, University of Utah, Salt Lake City, Utah 84112, USA.

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

Bert Hölldobler

On guard: hitchhiking minor-caste members of leaf-cutting ants defend the carrier against phorid attack.

enjoying all the social benefits of the colony. Others lead a more furtive existence and rely on their limuloid body form to minimize damage from attacks by host ants⁴. Phylogenetic analysis of these nest symbionts has only just begun, but it seems that the group includes several separate instances of adaptive radiation and numerous examples of convergent or parallel evolution³. Combined phylogenetic and behavioural analyses of symbionts and hosts should tell us a great deal about processes of adaptive radiation and

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Earth dons a different mantle

Raymond Jeanloz

THE past two decades have witnessed remarkable advances in experimentalists' ability to study materials at the conditions of the Earth's deep interior, and on page 170 of this issue, Duffy and co-workers¹ report the latest such step — the highest-pressure elastic-wave velocity measurements yet made for olivine, thought to be one of the chief constituents of the Earth's mantle. But instead of reassuring us by refining the details of well-established geophysical interpretations, the new data challenge our understanding of this region of the Earth.

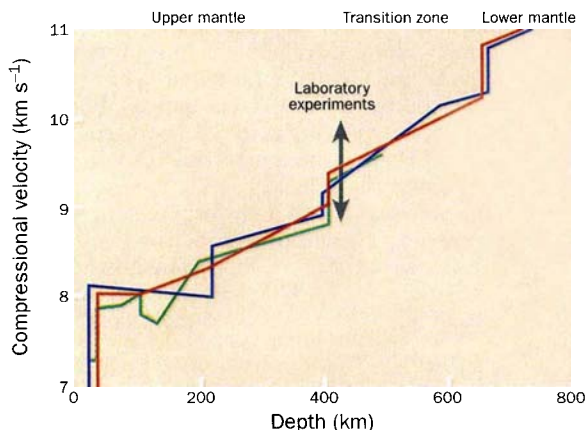
Olivine is widely accepted to be the predominant mineral in the topmost mantle: it is the primary mineral in rock fragments ejected volcanically from the interior, its elastic properties match the seismologically determined wave velocities of the upper mantle, and partial melting of olivine-rich rocks yields basalt, the most abundant type of lava erupted at the Earth's surface. On this evidence, the upper mantle is thought to consist of peridotite, a rock containing 50–60% (Mg,Fe)₂SiO₄ olivine.

Moreover, the rapid increases in seismic wave velocities that define the 'transition zone' in the mantle at depths of 410–660 km (see figure) are classically interpreted as being due to pressure-induced phase transitions in olivine. Just as graphite transforms to diamond at high pressures, olivine is known to transform to spinel and perovskite-type crystal structures at the pressures of the transition zone. Indeed, detailed comparisons between laboratory studies of the olivine phase transformations and the observed depths (hence pressures) of the seismological velocity jumps near 410 and 660 km depth yield key constraints on temperatures in the mantle².

The significance of the transition zone is highlighted by computer simulations revealing that the phase transformations strongly influence the pattern of mantle flow driving plate tectonics, and the related phenomena of earthquakes and volcanoes at the Earth's surface^{3–7}. Given its importance for understanding the Earth's planetary evolution, there is every incentive to increase our knowledge of this region in the mantle.

What Duffy and co-workers¹ have now done is to measure the elastic-wave velocities of Mg₂SiO₄ olivine right through the pressure range of the 410-km seismic discontinuity at the top of the

transition zone. The new results are in good agreement with independent measurements on (Mg,Fe)₂SiO₄ olivine to nearly the same pressures⁸. As the high-pressure elastic properties of the spinel-type 'beta' phase, to which olivine transforms, have also been determined over the past few years⁹, the new laboratory data offer the hope of making direct comparisons against the seismologically



How the velocity of compressional waves varies with depth in the Earth's upper mantle and transition zone, according to models derived from seismic measurements (PREM, ref. 14, blue line; iasp91, ref. 15, red line; ref. 13, green line). New laboratory measurements¹ show a velocity jump (indicated by the arrow) of 13% at about 400 km depth, far greater than these models suggest.

determined velocities through the transition zone.

Here is where the problem arises. The velocity jump associated with the olivine–spinel (beta phase) transition is 13% according to the laboratory data, yet the seismological observations reveal a jump of only 3–5% at about 400 km depth. At face value, the results imply that olivine makes up just 30–40% of the mantle rock, as little as half the amount of olivine traditionally thought to be present in the upper mantle. (Incidentally, this is a different issue from recent estimates that the lower mantle has nearly zero olivine component^{10,11}.)

One might object that the laboratory

data are collected at room temperature, rather than the temperatures of about 1,700–2,000 K relevant to the transition zone. However, although the individual seismic velocities depend on temperature, pressure and composition, the differences in velocity between olivine and beta phase are expected to be relatively insensitive to these variables. The seismic wave velocities depend primarily on density (as influenced by temperature, pressure and composition), so it is significant that the elastic properties of olivine have now been documented over the entire density range of the upper mantle¹.

The discrepancy between the observed and expected jumps in velocity near 400 km depth should give us pause. Do we really understand the bulk composition of the uppermost mantle, let alone the deeper reaches of our planet? Amplitude studies of seismic waves deflected from the transition zone have also raised questions of whether the phase transformations in olivine can really account for the observed structure^{12,13}.

In my view, it is not yet time to give up the traditional model of upper-mantle composition, although modifications are probably in order. The basic reasons for believing in a peridotite upper mantle, outlined above, still stand, but an olivine abundance in the range of 40–50% (rather than 50–60%) is probably to be preferred. Indeed, more primitive or 'fertile' peridotites (compositions capable of producing basalt on partial melting) tend to have greater pyroxene and reduced olivine contents. Also, the seismological observations are being refined: a recent study on the sharpness of the 410-km discontinuity invokes a velocity jump near 5.5% (corresponding to about 42% olivine)¹³, rather than the 2.5–4.5% earlier thought to be appropriate^{1,14,15}.

I further believe that texturing of the rock can influence the seismological observations at the level of detail at which they are now being interpreted. Just as the interface between crust and mantle is increasingly being recognized as a sheared structure (with tectonic deformations

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localized at, and thus enhancing, what is otherwise a compositional boundary), crystallographically preferred orientation may be associated with the phase transitions at depth. Far from it being a coincidence that the transition zone might be textured, computer modelling shows that it is a strong tendency for the phase transformations to modulate flow through the transition zone³⁻⁷. Texturing of the rock is thus a likely outcome, and may yield directional velocities well outside the bounds calculated for isotropic aggregates¹. Texturing may also help resolve some of the difficulties in reconciling seismic waveforms with the

equilibrium phase diagram for the olivine system^{12,13}.

Given the uncertainties in the acceptable olivine content of the upper mantle, the magnitude of the seismic velocity jumps and the possible effects of rock texture, a peridotite with 40–50% olivine seems to be our current best estimate. Perhaps it is geophysical arguments that ultimately yield the tightest constraints on the composition of the upper mantle? □

Raymond Jeanloz is in the Department of Geology and Geophysics, University of California, Berkeley, California 94720, USA.

SPATIAL LEARNING

The LTP–memory connection

Howard Eichenbaum

SINCE its discovery over twenty years ago¹, long-term potentiation (LTP) has captured the imagination of both cellular and behavioural neuroscientists as the kind of synaptic modification that is likely to support memory. LTP, like memory, is identified with hippocampal function, is induced rapidly, and shows a lasting incremental response to short bursts of activity at specific synapses — all consistent with a natural memory mechanism. But even these shared features cannot confirm a direct connection, and fresh attempts to draw the link are described in two papers in this issue (on page 182 by Bannerman *et al.*² and on page 186 by Saucier and Cain³) and in two others in *Neuron* by LeDoux and colleagues^{4,5}.

Those of us who follow this research are riding a roller-coaster between peaks of exhilaration over exciting new findings and troughs of disappointment when they are subsequently debunked. For example, a report that LTP-like increments in hippocampal synaptic efficacy result from a learning experience was quickly explained by a rise in brain temperature and other factors associated with any active exploratory behaviour⁶. Another claim that 'saturation' of hippocampal synapses by intense afferent stimulation blocks new learning could not be substantiated in typical learning situations⁷, showing that this approach too had its limitations⁸.

So what's the news today? Provocative though they are, the new findings have still not succeeded in halting the roller-coaster. On the downward track, two of the new reports^{2,3} undermine previous excitement over the behavioural pharmacology of LTP. Some of the most convincing evidence supporting a connection between LTP and memory came from an early demonstration that drug-induced blockade of NMDA-receptor-dependent hippocampal LTP selectively prevents new

learning⁹. And recent results from targeted genetic manipulations show that blocking the cascade of molecular triggers for LTP also causes severe memory impairment^{10,11}. But the two experiments reported elsewhere in this issue provide converging evidence that, whatever the pharmacological blockers do, they seem unable to abolish the central component of watermaze learning, that is, the encoding of a new spatial map.

The two studies^{2,3} have several parallels. Both are aimed at isolating new spatial learning by training animals in advance in swimming and other background skills. Furthermore, each shows that such pretraining protects against the otherwise detrimental effects of an NMDA-receptor blocker on new spatial learning. Between them, they also incorporate the pharmacologists' strategy of demonstrating the same pattern of behavioural effects using biochemically different drugs that share a common main effect on LTP.

The two groups diverge, however, in the interpretation of their results. Bannerman *et al.* conclude that hippocampal NMDA receptors may be critical for learning a spatial strategy but not for encoding particular maps. More sceptically, Saucier and Cain conclude that pretraining merely overcomes the motor side-effects of NMDA-receptor blockers by providing preliminary acquisition of swimming skills.

Perhaps they are both right. Consistent with a multiple memory systems perspective¹², NMDA receptors might be involved in separate brain pathways that support the acquisition of swimming skills and spatial strategies, and pretraining might allow both to be consolidated before drug treatment. Subtle differences in the type or extent of pretraining could account for why Saucier and Cain found non-spatial

experience was sufficient to prevent the effects of the NMDA-receptor block, but Bannerman *et al.* found that irrelevant spatial pretraining was necessary for the protection.

The present results do not exclude the possibility that new spatial learning is NMDA-dependent. Saucier and Cain's control data indicate asymptotic learning of a new environmental layout within just a few trials, suggesting that minimal residual capacity for LTP (in the dentate gyrus or elsewhere) might mediate the gradual acquisition of a spatial preference. Consistent with this, Bannerman *et al.* observed that new learning was slower when LTP was blocked. Although this account might bring the two interpretations together, the results bring us to the descent in the roller-coaster ride as they do not prove that hippocampal-dependent learning of a specific environmental map relies on NMDA receptors.

We are conveyed upwards, however, by new observations on the thalamus-to-amygdala circuit critical to auditory fear-conditioning. Rogan and LeDoux⁴ find that when high-frequency electrical stimulation of this pathway induces LTP, there is also an enhancement of synaptic responses to natural auditory stimulation. Furthermore, the same team has now shown that fear-conditioning enhances short latency sensory responses of neurons in the amygdala⁵.

These findings are illuminating in two respects. First, they support the view that there is nothing special about the hippocampus or spatial memory when it comes to LTP, consistent with the picture of multiple memory systems and loci for critical plasticity. Second, the approach taken by LeDoux and colleagues offers a potentially more decisive and therefore more fruitful way to link LTP and memory.

The magnitude, direction and longevity of increases in auditory evoked synaptic potentials paralleled those parameters for electrically induced synaptic potentials, showing us that natural information processing can make use of the very cellular

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and molecular mechanisms set in place by conventional, artificially induced LTP. Correspondingly, the observation of increased neuronal sensory responses that follow conditioning shows us that, like LTP, real learning can enhance information processing relevant to the task.

This approach of LeDoux and colleagues takes us beyond mere similarities between LTP and memory by bringing into contiguity the identical neural pathways and experimental procedures that

define LTP and sensory processing in memory. Focusing our efforts on neural processing, a level intermediate between the intracellular events and behavioural phenomena, could provide the most compelling evidence yet for the connection between LTP and memory. □

Howard Eichenbaum is in the Center for Behavioral Neuroscience, State University of New York at Stony Brook, Stony Brook, New York 11794-2575, USA.

MACROMOLECULAR PHYSIOLOGY

One small step for myosin . . .

Steven M. Block

THANKS to a growing arsenal of clever tricks, it has become possible to record the behaviour of individual biomolecules¹⁻⁷. Recent successes include the measurement of nanometre-sized steps and piconewton-sized forces produced by motor proteins such as kinesin and myosin, as well as the detection of single ATP turnovers. In pioneering work, Finer and co-workers⁴ accomplished direct recordings of the forces and displacements produced by heavy meromyosin (HMM), a two-headed proteolytic fragment comprising the business end of myosin, the protein responsible for muscle contraction. One central question that dogs the field is whether two heads are better than one — that is, whether a single head could be responsible for the observed motor properties, or whether participation by two heads is required. In the case of kinesin, current thinking favours cooperative, 'hand-over-hand' models⁸, although the issue is not settled.

On page 209 of this issue⁹, Molloy and colleagues address the question for myosin with an innovative optical trapping experiment, comparing the step sizes and forces produced by myosin subfragment one (S1), which consists of a solitary head domain, with those of double-headed HMM. In a study sure to fan the flames of controversy, they report that the average working strokes produced by these two species are roughly comparable, and significantly smaller than the value reported previously for HMM⁴ — about 4 nm, as opposed to 11 nm. At face value, their findings imply that myosin movement is fundamentally a single-headed affair. Moreover, their analysis suggests reasons why earlier step-size estimates may have been biased.

The new experiments were set up in much the same way as earlier work⁴, but with some novel twists, including an ingenious time-sharing method for manufacturing two traps from a single beam of laser light¹⁰. At the start, beads attached at each end of a single actin filament hover

about their equilibrium positions in two optical traps, which act as opposing optical springs, represented by harmonic potential wells (*a* in the figure; the mechanical equivalent is shown in *b*). Once contact between the actin 'tightrope' and myosin is established, the motor binds to the filament and — powered by the hydrolysis of ATP supplied in an appropriate buffer — takes a step.

Because the myosin is attached to a fixed support, it pulls on the filament and produces a brief deflection along the *x* direction, through a distance that presumably corresponds to the working stroke of the motor, provided that the traps are not so stiff as to impede this motion. Subsequently, the myosin releases the actin, whereupon the beads are drawn back towards their equilibrium positions. To measure force, the setup can be turned into an isometric clamp where the trap positions are servoed in such a way as to prevent the beads from moving. In this closed-loop mode, the feedback provides an output signal proportional to force.

In principle, once the apparatus is working it should only be necessary to record displacements (or forces) from many actomyosin interactions and analyse them. In reality, however, both the execution and interpretation of these experiments are complicated by brownian (thermal) motion and other considerations, such as the number of motor molecules on the surface of the large bead, which is difficult to control. At high surface densities, there looms the possibility of many heads contacting the actin filament, producing sustained interactions, large displacements, and other confounding phenomena.

The problem turns out to be more troublesome for myosin than for kinesin: with kinesin, its prolonged interaction with a microtubule makes it feasible to work with protein concentrations low enough to be confident of single-molecule behaviour^{1,3}. Arguments for single-molecule interactions in the actomyosin sys-

tem have been perforce less direct, and based largely on stereotyped features of the records at the lowest head densities^{4,11}. When working with S1, the problem becomes acute. Although single heads do function *in vitro*, they do so only at high surface densities, producing poor-quality movement at low speed^{12,13}.

One reason for this could be that myosin heads might prefer to act in pairs, and therefore a high density is required to produce near neighbours that can cooperate. Another might be that the shorter necks of single-headed constructs produce smaller steps. A third could be that single-headed constructs tend to attach to the substrate with unfavourable geometries, such that most heads are non-functional or sterically hindered. The finding by Molloy *et al.* that S1 and HMM generate similar steps argues against the second explanation and in favour of the third, although the first cannot be entirely discounted at the surface densities studied here.

Returning to the problem of brownian motion: the beads, of course, never remain stationary within the optical trap, but jiggle constantly. For the scheme of Molloy *et al.*, the physical treatment had to be simplified. Because the actin is much stiffer than the optical trap, the compound object consisting of the pre-tensioned filament with attached beads was idealized as two spheres connected by a rigid rod; that is, as a dumbbell. This ignores the possibility that a filament might behave more like a 'string' (inextensible, but infinitely compressible) than a 'spring', and it omits potential contributions arising from filament flexure or other series elastic elements at the bead junctions. Nevertheless, it seems a reasonable first approximation. A dumbbell behaves like a single particle suspended in a potential well of stiffness $\kappa_t = 2\kappa_{\text{trap}}$ (the two traps act as springs in parallel — *b* in the figure), so the displacement distribution should be gaussian, as confirmed by Molloy and colleagues. However, at the trap stiffnesses used here and previously, substantial thermal fluctuations persist that are of the same order of magnitude as the myosin working stroke.

This poses pitfalls for analysis. Imagine that the actin dumbbell fluctuated for an instant to the left through several nanometres, whereupon a myosin head happened to bind and abruptly pulled it further left. The apparent displacement, if scored merely by judging the bead's jump in position 'by eye', would be increased by the extra thermal drift. Conversely, if the bead fluctuated several nanometres to the right instead, but was again moved left by myosin, the opposing displacements would tend to cancel out (or even make the step negative), producing such a small change that it would be lost in the noise and not scored. The overall effect is that

only those jumps that are unidirectional and exceed some threshold are scored⁴, systematically biasing the estimate.

Molloy and co-workers reasoned that the true event distribution should contain both positive and negative displacements, and resemble a gaussian whose width is determined by thermal contributions (through the system stiffness), but whose centre is shifted from the origin by an amount equal to the step size. As smaller events are not readily apparent to the eye over background, the event histogram

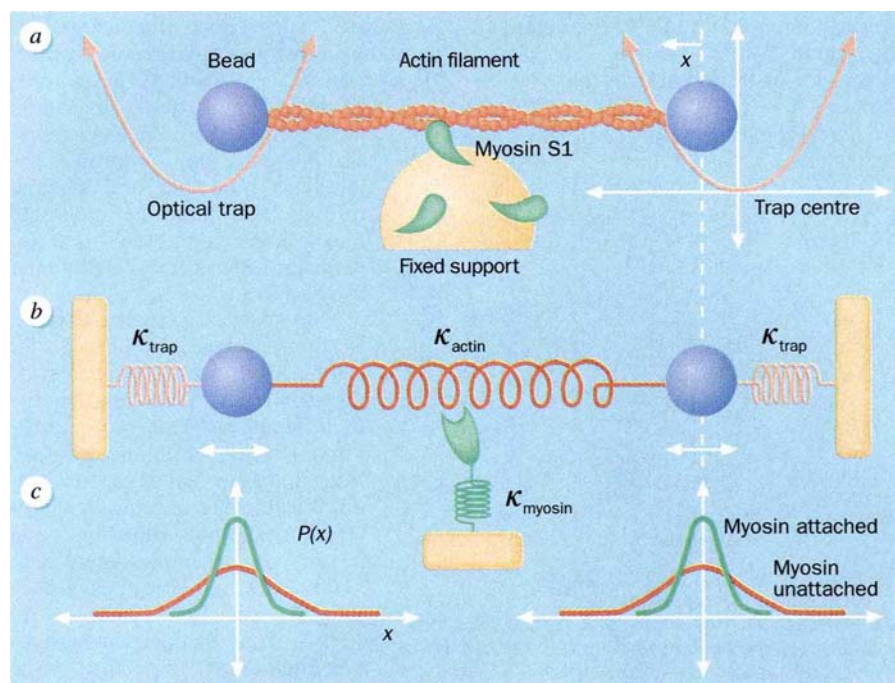
ditional stiffness to the system, κ_{myosin} , further reducing the noise when bound (panel c). The variance in displacement therefore supplies additional information about whether or not a myosin head is bound to the actin. By computing a running estimate of variance with time, Molloy *et al.* were able to focus attention on those portions of the records that contained small displacement events which had previously been ignored. Keying on the variance, they were able to reconstruct, by eye, most of the missing middles

be evidence of double-headed events, implying that some sort of intrinsic difference exists between S1 and HMM, resurrecting the notion that myosin heads cooperate. Second, additional effects may conspire to inflate the value of the working stroke for S1. The low-ish values for force obtained in the tension clamp mode (about 1.7 pN for both HMM and S1) led the authors to speculate that residual compliance in the large myosin-coated sphere may bias their estimate. This effect should be more pronounced at the high trap stiffnesses used in force-feedback mode, but it might also serve to reduce the measured steps at lower trap stiffness.

Another consideration is the possible effect of myosin head orientation. It has been argued¹⁵ that the step size for myosin depends on the relative angular orientation of the myosin head to the actin filament. A random array of heads, as was used here and previously⁴, would sample over all orientations and possibly underestimate the mean (but not the maximal) step size. Nevertheless, these would all constitute relatively minor corrections — at the end of the day, the estimate of step size may inflate, perhaps by a factor of two or so. Such a revised estimate, though larger than the value reported here, might not pose a very serious challenge to traditional notions of myosin movement¹⁶. We shall see.

The variance-based approach is clearly a step in the right direction. Although it does not circumvent all of the problems inherent in scoring events by eye, it raises the standard for single-molecule work. The next generation of experiments should benefit from improved instrumentation and more sophisticated methods of analysis that do not rely on human pattern recognition. □

Steven M. Block is in the Department of Molecular Biology, Princeton University, and the Princeton Materials Institute, Princeton, New Jersey 08544, USA.



Experimental geometry used to record single steps in the actomyosin system. *a*, An actin filament (red) is stretched taut by means of attached polystyrene beads (blue) of about 1 μm diameter, each held in one of two optical traps, depicted as harmonic potential wells (pink). The filament interacts with a myosin S1 molecule (green) bound to a third glass sphere (yellow) immobilized on the coverslip which serves as a fixed support. The equilibrium positions of the beads (dotted line) are displaced from the trap centres, reflecting the pre-tension in the filament. A photodiode sensor (not shown) records the x and y coordinates of one of the two spheres. *b*, Mechanical equivalent of the system in *a*, using the same colour scheme for corresponding elements. The optical traps function as weak springs attached to a stationary reference frame (yellow). The myosin crossbridge (green) acts as a spring of intermediate stiffness, and the actin filament (red) as a stiff spring (all stiffnesses are designated by κ). *c*, Variance in bead displacement. In the absence of myosin attachment, the position is distributed as a broad gaussian (red). Attachment of the myosin crossbridge stiffens the system and reduces the width of this distribution (green).

should resemble a displaced gaussian with a large portion of its centre missing. And that is precisely what they found. However, such histograms are rather unsatisfactory, as the data are weakest where they need to be strongest, near the peak of the distribution.

Here is where Molloy *et al.* got clever and were able to recover some of the 'missing' data. They observed that running noise levels in relatively less filtered displacement records dropped during the course of myosin-induced steps. The reason for this becomes apparent on inspection of *b* in the figure: the attachment of a myosin crossbridge introduces an ad-

ditional stiffness to the system, and the revised histograms resemble displaced gaussians whose centres have been shifted.

A myosin stroke as short as 4 nm is more readily accommodated by the sort of traditional, conformational-change model that has been proposed for myosin on the basis of the crystal structure¹⁴ of S1, but the story is unlikely to remain that simple. First, there is a hint of a second peak beyond 10 nm in the variance-based HMM data that is not seen for S1 (and which is not seen in the unreconstructed HMM data either for that matter). Given the limited statistics, this could represent a statistical fluctuation, but it might also

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Reduction of urban hazards

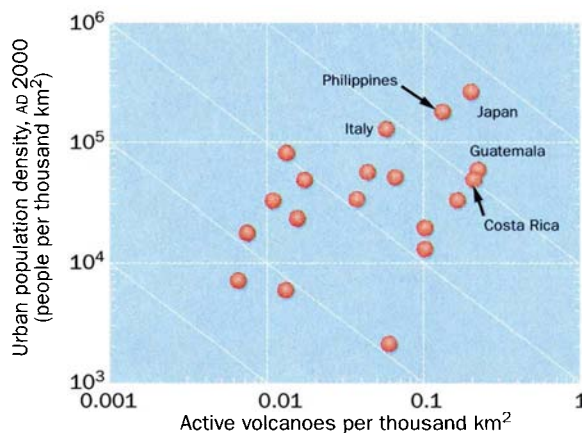
David M. Pyle

TWICE this century, large towns have been laid waste in minutes by volcanic eruptions. The fifty thousand resulting deaths in St Pierre, Martinique, and Armero, Colombia, account for over two-thirds of volcano-related deaths since 1900, and give an indication of the potent volcanic threat to the world's increasingly urbanized population. Within the next five years, half of the world's people and 70–90 per cent of those in middle- to high-income economies will live in towns and cities¹. Of these, at least 500 million will be living under the shadow of a volcano². So this, the International Decade for Natural Disaster Reduction, is an appropriate time to assess what can — or should — be done to reduce the hazard posed by volcanoes to densely populated regions. This was the theme of a meeting* to review progress on the 15 volcanoes targeted five years ago by the volcanological community with the aim of identifying and reducing hazards from future unrest. The principles³ that guided the selection of these 'Decade volcanoes' included a likelihood of eruption during the 1990s and a recognized hazard to local populations. Prominent conurbations lie just tens of kilometres from several of these volcanoes, including Seattle and Tacoma (close to Mount Rainier, USA), Manila (near Taal, in the Philippines) and the refugee camps at Goma (Nyiragongo, Zaire). Nine of the fifteen volcanoes have already erupted since 1990, causing the tragic deaths of tens of people including nine scientists.

Although volcanic hazard research programmes remain primarily in the hands of geologists⁴, the problem is of far wider significance. Areas of the Bay of Naples, overcast by the threateningly calm Vesuvius, have population densities approaching those of Hong Kong (F. Barberi, Univ. Pisa). In the event of a moderate-sized explosive eruption at Vesuvius, modelling shows that once the vertically directed fountain of ash collapses to form a hot ground-hugging particle-charged flow, it would be only a matter of five or six minutes before the crowded bayside apartments would be completely engulfed. This poses an enormous headache not only to the volcan-

ologists, but also to civil defence planners and public officials: how can one establish evacuation procedures that are going to work without causing civil strife, or exacerbating a disaster? Although focused research combining the geological know-how of volcanologists with the diverse skills from other disciplines represents the most obvious and clearly identified way forward, integrated studies remain thin on the ground.

A vivid illustration of the value of inter-



An indication of volcanic risk in selected countries can be gleaned by comparing projected urban population densities at the end of this decade with the areal distribution of volcanoes. Even a small explosive eruption (emitting 0.1 km³ of tephra) will have a severe impact over an area of 1,000 km². Unlabelled contours of risk, defined as the probability of eruption multiplied by the number of people affected, are shown for reference: each contour indicates an order of magnitude increase in risk towards the upper right hand corner. But although living in the shadow of a volcano represents a risk, volcanoes do provide benefits in the form of fertile soils and equable microclimates. Perceptions of risk may be limited by the typical human lifespan: between them, the 1,500 recognized active volcanoes typically have 50–70 eruptions of more than 0.1 km³ of rock per century⁵. For any individual living on the flanks of a volcano, this translates to less than a 5 per cent chance of an eruption of such a scale or larger during their lifetime.

disciplinary study arises from work by medical and geological scientists on the human effects of the ongoing eruption at Unzen, Japan, where a growing lava dome continues to shed cascades of incandescent debris down towards the town of Shimabara (P. Baxter, Univ. Cambridge; A. Neri and M. Todesco, Univ. Pisa). These flows can have calamitous consequences for those close to the volcano, but out on their margins they are survivable. Two prime factors cause death when engulfed in an ash cloud: inhalation of hot pulverized rock, and contact of uncovered skin with hot gas. Simply wearing clothing that covers the arms and legs will halve burns to 20 per cent of the body area, and reduce the deaths in a typical population

by a factor of three. Remaining indoors, or turning to face away from an approaching ash cloud, would be similarly effective in reducing ash inhalation and improving one's chance of survival.

Numerical simulations of volcanic eruptions have the great advantage that the temperature and ash concentration can be tracked as a function of eruption size, and allow refined assessments of the actual risk posed by future eruptions to real people and existing structures. Similar models can be used to assess how man-made barriers might impede the passage of, and reduce the devastation caused by, these same pyroclastic currents (F. Dobran, GVES, Rome). The benefits of hazard mitigation — measured in hypothetical lives and property saved — can be quantified by intelligent modelling, and information like this should be integrated into the developing civil defence plans for urban centres threatened by volcanoes.

Some particular problems faced by the developing economies of southeast Asia are exemplified by the problems at Taal volcano in the Philippines (E. Listanco and R. Punongbayan, Philippines Inst. Volcanology). Taal is now recognized as the site of multiple large-volume eruptions (ejecting some 50 km³ of rock), as recently as 5,000 years ago. Little is known about the way in which this volcano works, or of the particular signs which mark an impending large eruption, but even a repeat of the small 1966 eruption would have severe effects on the growing urban communities, already numbering over a million people, that flank the flooded caldera. Despite the volcanological work remaining, the recognition outside the scientific community of the potential risks, especially to the growing tourist traffic to Volcano Island, which includes the site of the 1966–70 eruption, has led to collaboration between scientists and planners which has yet to be matched at many of the other Decade volcanoes.

The acute problems facing urban planners are, if anything, worsened in some industrialized nations because Western concepts have shifted from the idea of living at the mercy of nature, to that of controlling nature to suit human needs, and of seeing natural phenomena —

*Volcanoes in Town, IAVCEI (International Association of Volcanology and Chemistry of the Earth's Interior) Conf. on Volcanic Hazard in Densely Populated Areas, Rome, 27 September–1 October 1995.

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volcanic eruptions included — as a focus for tourism and recreation (J. Lockwood, Hawaii Volcano Observatory; J. Rhodes, Univ. Massachusetts). Billions of dollars have been poured into developing tourist infrastructures on the flanks of Mauna Loa since the 1984 eruption, even though many of these complexes lie within areas previously enveloped by active lava, and still at risk from future eruptions.

Earlier Polynesian societies had a deeper appreciation of the extraordinary powers of the volcano, less firmly anchored roots, and more willingness to

relocate in the event of an eruption, in contrast to the technologically 'advanced' and yet highly dependent societies of today. There are clearly many important lessons to be learned, but until attempts to define and reduce volcanic hazards engage the wider community and not volcanologists alone, the wrong questions may be asked and inappropriate solutions presented. □

David M. Pyle is in the Department of Earth Sciences, University of Cambridge, Downing Street, Cambridge CB2 3EQ, UK.

CLIMATE SYSTEM

Driving the ocean conveyor

Andrew J. Weaver

ONE of the central questions in understanding past and future climates concerns the North Atlantic conveyor and how it might respond to increasing anthropogenic greenhouse gases and changes in the net flux of fresh water into its basin. As he reports on page 145 of this issue¹, Rahmstorf has now undertaken a detailed study of the sensitivity of the conveyor to changes in freshwater input, using a global ocean model coupled to a very simple atmospheric model. His results suggest that the present-day conveyor may be less stable than many researchers have thought. He further suggests that a continual $0.06 \times 10^6 \text{ m}^3 \text{ s}^{-1}$ of additional fresh water (about a quarter of the discharge rate of the Amazon river) into the North Atlantic could irreversibly shut down the conveyor with a pronounced cooling effect on the surrounding areas.

In the North Atlantic, the ocean acts as a large-scale conveyor that transports heat from low to high latitudes. Most of the oceanic heat transport in the North Atlantic is thought to be associated with the thermohaline circulation (that part of the ocean's circulation driven by fluxes of heat and fresh water through the ocean's surface). In the present climate, high-latitude cooling together with low-latitude heating accelerates the thermohaline circulation with poleward flow at the surface. Treating the ocean conveyor as roughly analogous to an automobile, we can consider the thermal forcing to act as the accelerator pedal. On the other hand, net high-latitude precipitation, run-off and ice melt, and low-latitude evaporation, tend to apply the brakes on the circulation.

In today's North Atlantic the thermal forcing dominates over the haline (fresh-water) forcing and the conveyor belt moves forward with deep water forming in the Greenland, Iceland and Norwegian (GIN) seas through intense heat loss to the overlying atmosphere. In addition, the Labrador Sea is a source of deep water

(LSW) formed through intense winter cooling which overrides and mixes with the denser GIN sea waters as this body of water, collectively termed North Atlantic Deep Water, flows towards the Equator.

No such deep sinking exists in the Pacific. If one compares the climate of Bodö, Norway, with average January temperature of -2°C and average July temperature of 14°C , to that of Nome, Alaska, with average January temperature of -15°C and average July temperature of 10°C (cities at similar latitudes and on the western flanks of continental land masses) one directly sees the impact of the poleward heat transport of the thermohaline circulation. Clearly, then, any interruption of the conveyor would have considerable effects on the climate of Europe and the North Atlantic region in general.

Rahmstorf began by integrating his global model under present-day forcing to an equilibrium climate with about 20 Sv ($1 \text{ Sv} = 10^6 \text{ m}^3 \text{ s}^{-1}$) of North Atlantic Deep Water formation (see figure). He then applied an external perturbation which slowly increased the amount of fresh water into the North Atlantic at a rate of 0.05 Sv per year. As expected from our automobile analogy, as the brake was depressed the conveyor slowed down. At a critical value of about 0.03 Sv, convection in the Labrador Sea shut off permanently. If at this stage the perturbation was slowly reversed at the same rate then point 2 on the figure was reached, which has the same forcing as point 1 but with a different strength of the conveyor. If instead the brake was continually applied then eventually, once the perturbation reached about 0.1 Sv, the conveyor completely halted. Rahmstorf also reversed the perturbation from this stage and reached point 3 when present-day forcing was recovered.

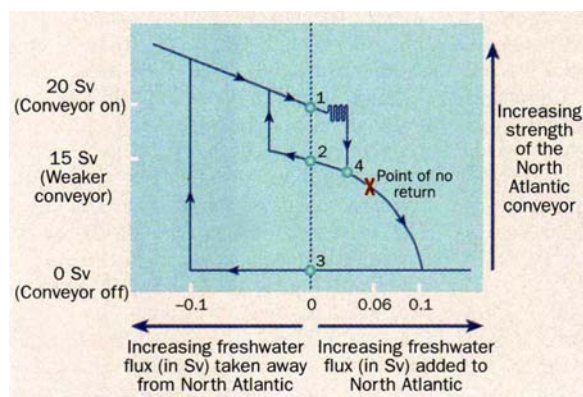
Thus, under present-day forcing, Rahmstorf has found three possible configura-

tions of the North Atlantic conveyor: on, off and on with no LSW. To look at it another way, the automobile has at least three gears and so operates at three speeds under the same pressure on the accelerator and brake pedals. The conveyor, like the automobile, rapidly jumps and jolts between speeds as the gears are changed. The existence of three equilibria of the North Atlantic conveyor is not in itself a new result (see refs 2 and 3) but the beautiful illustration of the transition between these equilibria associated with small changes in the hydrological cycle is new.

States very close to the present day are recovered as one traces around the 'hysteresis' curve of the figure. Of additional importance is the experiment in which Rahmstorf determined the 'point of no return' for the North Atlantic conveyor (X on the figure). If the forcing was kept steady at 0.05 Sv of extra fresh water into the North Atlantic, then the weakened conveyor (with no LSW formation) remained stable. This was not the case when the extra freshwater flux forcing was retained at only 0.06 Sv (and the conveyor had a strength of 12 Sv). In this case the conveyor eventually ground to a halt. A further remarkable result is the illustration of a regime (in the figure, just before the point where LSW ceases) where no steady circulation is possible and the system oscillates with interdecadal timescale.

What do these model results have to say with respect to the real world and are they reliable? Let us first focus on the prediction that LSW formation shuts down with an additional 0.03 Sv of fresh water into the North Atlantic (about twice the discharge of the St Lawrence River). The North Atlantic actually has a historical example known as the Great Salinity Anomaly⁴ which caused LSW to cease from about 1968 to 1972⁵. From ref. 6 we can estimate the magnitude of the Great Salinity Anomaly perturbation to be about 0.032 Sv for a period of 2 years (strikingly similar to the number found by Rahmstorf). Of course, when the source of fresh water (Arctic ice export⁷) was removed, LSW formation recovered.

The most worrying prediction is the 'point of no return' of only 0.06 Sv for the sustained perturbation of the freshwater forcing. Recent results⁸⁻¹¹ from coupled atmosphere-ocean modelling aimed at understanding the climate response to increasing greenhouse gases have all shown that, as the climate warms, the northward transport of water vapour in the atmosphere is enhanced, thereby increasing the supply of fresh water to the North Atlantic Ocean. In addition, one model⁹ reveals that the thermohaline circulation initially weakens but eventually recovers if the forcing is held fixed once the atmospheric concentration of CO_2 reaches twice its present level. This is not the case in the response under quad-



Schematic diagram showing the behaviour of the North Atlantic conveyor as a freshwater perturbation is slowly varied. The abscissa shows the size of the perturbation applied while the strength of the conveyor is represented in the ordinate. Point 1 indicates the present-day conveyor strength under present-day forcing. Points 2 and 3 indicate two other possible configurations of the conveyor under the same forcing. Point 4 indicates where the formation of Labrador Sea Water ceases while the X marks the 'point of no return', beyond which the conveyor inevitably shuts down. Interdecadal oscillations of the conveyor occur just before the point where Labrador Sea Water formation shuts down.

rupting of CO_2 forcing⁹, although it appears that the deep ocean may not have fully equilibrated in this latter experiment. These results, together with Rahmstorf's analysis, suggest that greenhouse-gas-induced warming over the North Atlantic and Europe would be smaller (perhaps even a net cooling) than it would be if the thermohaline circulation were to remain active. The effects on the ocean environment are probably far-reaching but not quantifiable at this stage.

On page 165 of this issue Manabe and Stouffer¹² try to quantify the response of the coupled atmosphere-ocean system to large perturbations of fresh water associated with the melting of continental ice sheets during the transition from the last glacial period to the present interglacial period. By adding a large perturbation of 1 Sv of fresh water uniformly released over the 50°–70° latitude band of the North Atlantic for a 10-year period, they show that the thermohaline circulation does indeed weaken but appears to recover (after a few oscillations) by year 300 of the simulation.

How can we relate this seemingly conflicting result to the work of Rahmstorf? Closer inspection reveals subtle differences between the independent experiments. Rahmstorf maintains his much smaller freshwater flux perturbation for a long period of time whereas Manabe and Stouffer apply their perturbation over a rapid 10-year period — too short a time to "invoke an advective spindown of the circulation", in the words of Rahmstorf. This spindown timescale is of the order of a few centuries and is linked to the timescale of the overturning of the North Atlantic conveyor. An extremely interesting undertaking would be to re-

run Manabe and Stouffer's glacial runoff experiment, reducing the perturbation by a factor of 10 but increasing the time over which it is applied by a factor of 10. The net freshwater perturbation would have the same size but it would persist over a longer period, perhaps causing a shutdown of the North Atlantic Conveyor as suggested by Rahmstorf's results.

Rahmstorf has put many pieces of the climate puzzle together. He has demonstrated the existence of multiple modes of operation of the conveyor under present-day forcing; the existence of rapid transitions between these modes; and the existence of unstable, variable regimes. Most importantly, his

work graphically illustrates where our present climate sits on the stability curve. Combined with results from previous studies, it sends out a warning about the possible response of the North Atlantic conveyor to increasing atmospheric greenhouse gases.

Nevertheless, a cornerstone of the puzzle is missing. That is the corroboration of these results using fully coupled atmosphere-ocean models which resolve the annual cycle, do not drift away from the present-day climate and do not need to employ flux corrections to prevent this drift. As pointed out by Rahmstorf, atmospheric variability may blur the distinction between similar ocean equilibria. On the other hand, it might cause more frequent transitions between these equilibria. Adequate models are perhaps a few years away, as is the computer technology which will allow them to be integrated many times for thousands of years. □

Andrew J. Weaver is in the School of Earth and Ocean Sciences, University of Victoria, PO Box 1700, Victoria, British Columbia V8W 2Y2, Canada.

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Dyes born again

LAST week Daedalus invented a universal paint. UniPaint contains a wide range of dyes. Exposure to coloured light bleaches the dyes that absorb that colour, leaving the ones that reflect it. It therefore takes up the imposed colour, which is sealed in when the paint sets.

Daedalus now wants to unseal the colour. The photobleaching of a dye molecule is reversible — as shown by the Q-switched laser. If UniPaint's fugitive dyes could be unbleached, it could be restored to its primal black. A different subset of dyes could then be bleached away, giving a different colour. The process could even be repeated, allowing the colour to be updated endlessly. DREADCO's chemists are now attacking the problem. UniPaint traps the bleached form of each dye in an oxidized state, from which it could be reduced again by bombardment with electrons.

The simplest way would be to expose the paint to a β -emitter such as tritium; but the commercial process will probably use a negative corona discharge. A new colour, or even a complex coloured image, could then be projected onto the restored paint, and fixed either by air oxidation, or by spraying positive charge. When the chemistry has been perfected — and it will be at least as complicated as colour film — DREADCO's 'RePaint' should take the market by storm.

Armies will welcome RePainted tanks, guns and uniforms that take up any camouflage pattern projected onto them, easily updated for any theatre of operation. Motorists will impress the Joneses, and thieves and criminals will baffle the police, by rapidly and repeatedly changing the colour of their RePainted vehicles. Home owners will also rush to RePaint their property. A special projector will image the craziest colours and patterns on their walls and even their clothes, and erase them again at will. Best of all, printing will be transformed. These days we all face a rising tide of pamphlets, magazines, junk mail and other ephemeral multicoloured bumf. To stop this waste of paper, DREADCO will issue sturdy, well-bound blank books, pamphlets, papers and journals, each page uniformly coated with 'ReInk'. The publisher will project his text and pictures straight off the computer screen onto their receptive pages. When the reader has finished with the product, DREADCO will pay a small return fee for it, erase it, and sell it to the publisher again. The endless trivial literature that threatens to bury us will be neatly recycled, leaving permanent printing for the few publications of enduring importance — such as *Nature*.

David Jones

A fly's ultimate con

SIR — Many aberrant insects are known from colonies of social insects¹, but few as bizarre as that reported here. In the rain-forest of Malaysia, in 1994, two of us (A.W. and U.M.) collected an entire bivouac of a driver ant, an undescribed species of *Aenictus* Shuckard, situated in the fork of a tree. It was formed by ants that had interlocked their tarsal claws to form a temporary nest-like structure. From this bivouac, one of us (K.R.) recovered 104 specimens of an unidentified insect (Figs 1, 2), 80 Diptera larvae of the family Phoridae, 57,100 worker ants and a single queen. The latter was in a non-physogastric state, indicating that the colony was in its migratory phase.

The unidentified insects were flightless, legless (apart from reduced coxae and trochanters), and had vermiform abdomens lacking tergites 2–7, but bearing strong setae. Despite the larviform appearance, the structure of the head and the abdominal terminalia indicated that they are adults which closely resemble the larvae of their ant host. The form of the proboscis, antennae and thorax (Fig. 1a) immediately excluded them from Hymenoptera and identified them as Diptera.

After failing to match these flies to any known myrmecophilous genera, a sample was sent to David Kistner (California State University, Chico), who, in collaboration with A. and M. Newton (Field Museum of Natural History, Chicago), concluded that they might be highly aberrant Phoridae. This was confirmed by one of us (R.H.L.D.) from the structure of the antennae and clypeo-cibarium.

Many myrmecophilous and termitophilous Phoridae possess flightless

females whose wings are either absent or reduced, or whose membranes are shed when the fly enters its host's nest. They are normally transported by the males to the colonies of their hosts during mating². All these aberrant phorids possess legs. Our specimens are all mature females, gravid with eggs (Fig. 2a). These eggs are also examples of mimicry, closely resembling those of their ant host. The presence of sperm, in a spermathecal pocket discharging into the atrium, indicates that the species has males.

Vestigial legs are previously unrecorded in Phoridae, and are exceedingly rare in adult Diptera. In the bat parasites *Ascodipteron* Adensamer (Streblidae), the newly emerged females possess both wings and legs, but, on invading their hosts, they shed the wings and the legs beyond their trochanters³. The closest parallel to our bizarre phorid is the marine midge *Pontomyia* Edwards (Chironomidae), whose females possess vermiform abdomens and lack wings, halteres and front legs at the time of emergence. Their middle and hind legs are vestigial, being reduced to coxae, trochanters and, in some cases, abbreviated femora as well. In one species there are also vestigial tibiae^{4,5}.

In our phorid, the middle and hind coxae are the most developed, but the endoskeleton associated with these coxae is not fully developed. Thus, the metafurca is not fully developed. The metafurca is not fully sclerotized ventrally and lacks any sclerotized connections with the mesofurca (Fig. 2b). Such an incomplete endoskeleton would be unlikely to allow proper functioning of the legs if they were still present. Similarly, the exoskeleton of the thorax shows incomplete sclerotization and seems to be insufficiently fused to allow normal flight or walking. Thus, the mesonotal and metanotal phragmata are not fused, so that the latter appears detached. The only representatives of wings are the tegulae, basicostas and roots of the costas (Fig. 1a). The halteres are vestigial, with uninflated knobs. These reduced structures represent a striking case of heterochrony, a phenomenon associated with other aberrant Phoridae and most strongly manifested by some of the termitophilous Termitoxeniinae^{2,6}.

This phorid will be assigned to a new genus and fully described else-

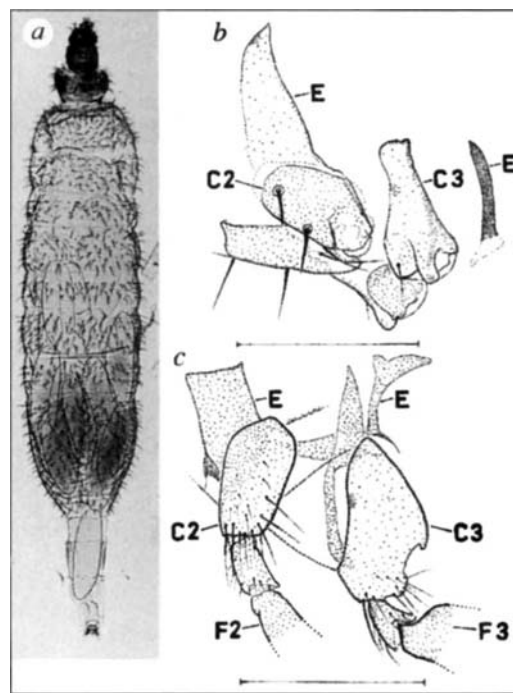


FIG. 2 Legless fly and its leg rudiments compared with those of a normal phorid. a, Whole fly showing mature eggs within. b, c, Middle and hind coxae of legless phorid fly (b) and *Metopina oligoneura* (c). C2, middle coxae; C3, hind coxae; E, endoskeleton (including mesofurca to left and metafurca to right); F2, base of mid femur; F3, base of hind femur. Scale bars, 0.1 mm.

where⁷. Evidently, the flies and their larvae are fully integrated into the ant colony and totally deceive their hosts. Both must be producing pheromones that mimic those of their host. In standard keys to insect orders⁸, the lack of legs would cause these adult flies to be identified as anomalous larvae of Diptera or other endopterygote insects. It is probable, therefore, that others have collected these highly aberrant flies, but have been unsuccessful in identifying them*.

A. Weissflog

U. Maschwitz

Zoologisches Institut,
Johann Wolfgang Goethe-Universität,
60054 Frankfurt am Main, Germany

R. H. L. Disney

University Department of Zoology,
University of Cambridge,
Cambridge CB2 3EJ, UK

K. Rościszewski

Staatliches Museum für Naturkunde,
Karlsruhe, D-76042 Karlsruhe, Germany

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*If others have collected similar legless flies but failed to identify them to their order or family, R.H.L.D. would be pleased to examine such specimens.

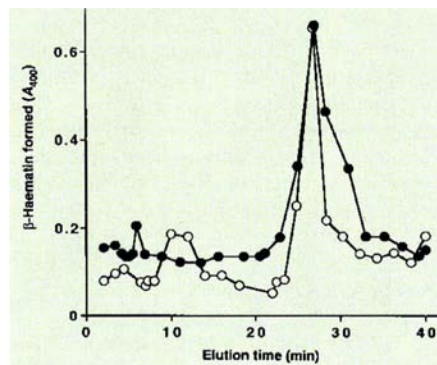


FIG. 1 Left face of legless fly. a, Head and thorax; b, whole fly.

Haem polymerization in malaria

SIR — Haem that is derived from the breakdown of host cell haemoglobin by malarial parasites is sequestered by the protozoan as the polymer haemozoin. Dorn and colleagues have reported an uncharacterized 'haem-derived material', associated with both authentic haemozoin and experimentally polymerized haematin, which accelerates haem polymerization¹. This active principle is extractable with acetonitrile, resistant to destruction by proteolysis or heat, and inhibitable by antimalarial drugs such as chloroquine. We report here that an identical activity is associated with phospholipids contaminating both haemozoin and β -haematin preparations. These lipids promote the rapid polymerization of haem, and may play a role in the formation of haemozoin by the malarial parasite.

During parasite maturation, more than 75% of host cell haemoglobin is catabolized, liberating potentially toxic haem at high rates². This free haem is detoxified by parasite-specific polymerization, resulting in the inert polymer haemozoin, which forms large insoluble crystals within infected erythrocytes. The molecular mechanism of this polymerization has remained elusive, but native haemozoin formation was previously conjectured to be catalysed by a parasite-dependent activity, consistent with a hypothetical 'haem polymerase' enzyme³.



Haem-polymerizing-activity profile of HPLC fractions from acetonitrile extracts of experimentally synthesized β -haematin (open circles) and malarial haemozoin (filled circles). Acetonitrile extracts⁴ from native haemozoin (*P. falciparum*, clone HB3) and β -haematin were concentrated and further separated on a silica column by normal-phase HPLC with a hexane/isopropanol solvent system. Individual fractions were concentrated to dryness and solubilized in 500 μ l (haemozoin) or 50 μ l methanol (β -haematin). A 50- μ l aliquot of each fraction was assayed for haem-polymerization activity by overnight incubation at 37 °C in 1 ml 0.165 M acetate pH 5, 50 μ M haem, 1.1 mM methanol. The resulting haem polymer was precipitated at 12,000g at room temperature (20 min), washed twice with 0.1 M NaHCO₃ and measured photospectroscopically at 400 nm after solubilization in 1 ml 0.1 M NaOH (ref. 5).

Acetonitrile extracts of authentic haemozoin, free of detectable haem, cause rapid haem polymerization⁴. Separation of the acetonitrile-soluble material from native haemozoin by normal-phase high-pressure liquid chromatography (HPLC) with a hexane-isopropanol gradient revealed a rather broad peak that was active in the polymerization of reagent haem (see figure). Active fractions had no absorbance at 400 nm and absorbed only weakly at 210 nm, confirming the absence of porphyrins. The active fractions contained significant amounts of organically bound phosphorus and could be cleaved with phospholipase B. Treatment of material derived from acetonitrile extracts of haemozoin with water-free methanol/potassium hydroxide at 80 °C yielded several active fractions with different chromatographic properties which were identified by mass spectrometry as the methyl esters of oleic, palmitic and stearic acids.

A similarly active fraction with phospholipid-like chromatographic properties could be readily extracted (at low yield) from β -haematin chemically synthesized from commercially available haem (see figure). Commercial haem is typically prepared by extraction from intact erythrocytes, so the presence of these lipid contaminants may not be particularly surprising. In fact, much more haem-polymerizing activity was present in acetonitrile extracts of β -haematin that had been chemically synthesized in the presence of small amounts of dioleoyl-phosphatidylethanolamine (ratio haem/phospholipid: 100/1).

While phospholipids may account for the acetonitrile-soluble, heat-stable and protease-resistant haem-polymerizing activity described by Dorn *et al.*¹, it is unclear whether the polymerization of haem within malaria-infected erythrocytes is effected solely by this apparent activity of endogenous lipids, or rather depends on the action of an as yet unidentified haem polymerase enzyme. These are important issues, as the sequestration of

haemoglobin-derived haem may be critical to the survival and successful maturation of the malarial parasite, and may represent an important target for antimalarial drugs.

Klaus Bendrat
Bradley J. Berger
Anthony Cerami

*The Picower Institute for Medical Research,
350 Community Drive,
Manhasset, New York 11030, USA*

RIDLEY *ET AL.* REPLY — Haem polymerization was first observed *in vitro* using parasite extract and was assumed to be caused by a 'haem polymerase' enzyme³. Later, this activity was found to be solely dependent on the presence of pre-formed haemozoin and to occur in the absence of protein¹. There was no need to invoke an enzyme to account for haem polymerization. It was also observed that haem polymerization can be initiated, in the absence of pre-formed haemozoin, by the addition of an acetonitrile extract of *Plasmodium falciparum*⁴. At first thought to be a source of 'haem polymerase' enzyme, the activity of the extract was also shown not to be mediated by protein¹. Bendrat and colleagues now suggest that this latter activity is in some way mediated by lipids.

Their work is a useful addition to the debate on this process, and their conclusion seems reasonable, though how the initiation of haem polymerization occurs mechanistically remains uncertain. Whatever the mechanism, we agree with Bendrat and colleagues that the activity observed in the acetonitrile extracts is not specific to parasite material. Acetonitrile extracts of uninfected erythrocytes will also initiate haem polymerization (A.D., unpublished results). The question remains, why does haemozoin, a regular crystalline polymer, form so readily in the parasite? To answer this question, it is necessary to understand the structure of haemozoin and to appreciate the role of pH in this process.

Haemozoin is believed to consist of polymeric haem linked by an Fe³⁺-O bond between the ferric ion of one haem moiety and one of the two propionic acid side chains of an adjacent haem moiety^{6,7}. These polymeric strands are further linked to each other by inter-strand hydrogen bonding between the remaining free propionic acid side chains⁷. The formation of this structure requires conditions in which 50% of the propionic acid side chains are deprotonated, which occurs at pH values equal to the pK_a of haem. We have measured this to be 4.9±0.1 (M.K., unpublished results), which coincides with the pH of the parasite food vacuole⁸, the organelle within which haemozoin formation occurs. It also coincides with the pH optimum measured for the *in vitro* polymerization process initiated by acetoni-

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trile extracts (A.D., unpublished results). Thus, an appropriate pH and an environment allowing initiation of polymerization, possibly involving lipid, can account for the formation of parasite haemozoin.

Bendrat *et al.* leave open the possibility that a haem polymerase enzyme may yet be found to exist in the parasite. We have previously postulated that protein(s) may serve as a structural focus about which the

initiation of haem polymerization might occur¹. However, we stress that there is no evidence yet for a haem polymerase enzyme as such, and that there is no need to invoke one to explain the currently available data.

**Robert G. Ridley, Arnulf Dorn
Hugues Matile, Manfred Kansy**

*Pharma Division, Preclinical Research,
F. Hoffmann-La Roche,
CH-4002 Basel, Switzerland*

UK lake plankton and the Gulf Stream

SIR—Recent studies suggest that plankton succession in temperate lakes is strongly influenced by year-to-year variations in the weather¹. Here we show that the inter-annual variations in the average summer biomass of zooplankton in Lake Windermere between 1966 and 1991 were closely correlated with north-south displacements of the Gulf Stream in the western Atlantic. The mediating factor seems to be subtle changes in the early summer weather which influence both the onset of thermal stratification and the growth of edible algae. Whereas similar responses can be seen in the seas around the European Shelf², Lake Windermere seems to be a particularly striking integrator of climatic events.

Lake Windermere is the largest lake in the English Lake District (54° 22' N, 2° 56' W), and is divided into two distinct basins by a large island. Temperature profiles have been recorded in the North Basin (area 8.05 km², mean depth 25 m, maximum depth 64 m) since 1935 and samples of crustacean zooplankton collected at fortnightly intervals since 1940. Monthly charts of the position of the north wall of the Gulf Stream have been published for the period 1966–91, based on data from surface, aircraft and satellite observations. An index of Gulf Stream latitude was constructed from these charts using principal components analysis².

Correlations between marine plankton abundance and the position of the Gulf Stream seem to be related to local variations in the intensity of thermal stratification^{2,3}, so similar effects might be anticipated in inland waters on the European Continental Shelf. There is a strong negative correlation between the summer biomass of zooplankton in the

North Basin of Lake Windermere and the latitude of the Gulf Stream (see *a* in the figure). An important factor influencing the seasonal dynamics of zooplankton in Lake Windermere is the timing and intensity of thermal stratification⁴. When the lake becomes stratified in early summer, the growth of edible algae occurs before the zooplankton are able to lay their full com-

onset of stratification was obtained by combining three measurements from early June profiles: the depth of the maximum temperature gradient, the rate of deepening of the 9° isotherm and the difference in depth of the 9° and 10° isotherms. These measurements were combined to form the index by subtracting the mean, dividing by the standard deviation, and then subtracting the last measurement (which decreases with intensity of stratification) from the sum of the first two (which increase with intensity of stratification).

The relationship between the biomass of zooplankton and this empirical index of stratification (*b* in the figure) shows that when the lake becomes thermally stratified in early summer, the biomass of zooplankton is much lower than when the lake becomes stratified later in the season. The year-to-year variations in thermal stratification of Lake Windermere are in turn closely correlated with the north-south movements of the Gulf Stream (part *c* in the figure). When the Gulf Stream is displaced northward, the early summer thermocline is shallower and better defined than when the Gulf Stream moves south. Similar relationships have been recorded in other thermally stratified water bodies. The stratification calculated for Esthwaite Water, a much smaller and shallower lake in the same area, is also shown in part *c* in the figure.

We believe that the results reported here and in the marine plankton demonstrate a hitherto unsuspected link between weather patterns around the United Kingdom and oceanic events at the other side of the Atlantic⁵. In agreement with this, long-term changes in the productivity of above-ground vegetation at Bibury, Gloucestershire, UK, have been shown to correlate with Gulf Stream northerliness⁵. In all these cases the signal appears more strongly in the biological data than local meteorological observations. The data from Lake Windermere have the advantage of indicating how weather variations can be integrated by physical and biological processes, and may provide a sensitive measure of regional climate change.

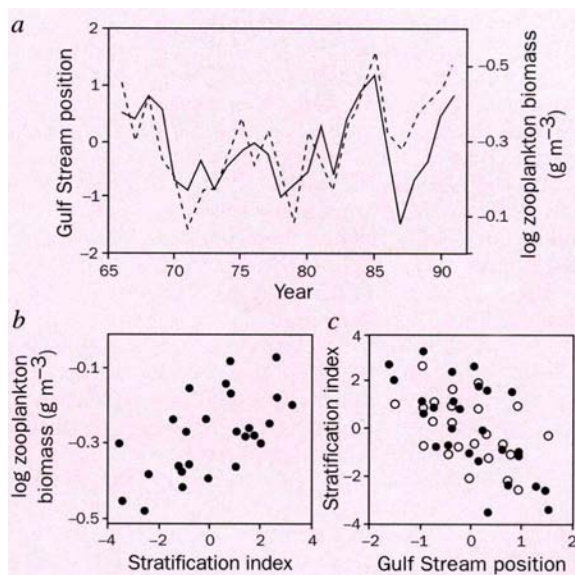
D. G. George

*Institute of Freshwater Ecology,
The Ferry House,
Ambleside, Cumbria LA22 0LP, UK*

A. H. Taylor*

*Plymouth Marine Laboratory,
Prospect Place,
West Hoe, Plymouth PL1 3DH, UK*

*To whom correspondence should be addressed.



a, Logarithm of the mean summer (May–September) biomass of zooplankton in the North Basin of Lake Windermere (inverted, solid line) compared with the latitude of the north wall of the Gulf Stream (broken line). A linear trend has been removed from the biomass data. *b*, Scatter plot relating zooplankton abundance in Lake Windermere to the index of stratification. The zooplankton samples were collected and analysed as in ref. 6. Correlation coefficient, *r*, 0.59. *c*, Scatter plot relating the index of stratification to the position of the north wall of the Gulf Stream. Closed circles show data from Lake Windermere, open circles those from Esthwaite Water. Calculations for Esthwaite Water used the 11° and 12° C isotherms. Values of *r* are 0.65 and 0.56, respectively, for Lake Windermere and Esthwaite Water. Each *r* value has *P* < 0.01 after allowing for serial correlation (as in ref. 3).

plement of eggs, whereas if the lake becomes stratified later in the summer, the growth of edible algae is delayed and there is a better match between the zooplankton and their food.

These effects of early summer stratification can be illustrated by analysing the thermal characteristics of the lake at the beginning of June. A general index of

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Gene duplications in *H. influenzae*

SIR — The sequences and structures of proteins characteristic of late evolution indicate that most, and possibly all, have arisen through a process of gene duplication, mutation and, in some cases, recombination¹. At some point, this process became more important for the evolution of modern organisms than the *ab initio* creation of new proteins. The recent sequencing of the complete genome of the bacterium *Haemophilus influenzae*², which identified some 1,680 protein sequences (two-fifths of the number expected for *Escherichia coli* and about one-fortieth that in humans), has allowed the estimation that at least one-third of the proteins in this small bacterial genome arose from gene duplication.

We carried out an all-against-all comparison of the *H. influenzae* protein sequences using the Smith–Waterman algorithm³. Although this is the most accurate method for comparing pairs of sequences⁴, calculations of this kind have intrinsic limitations. Proteins can diverge to a point where their common evolutionary origin and function cannot be seen from simple sequence comparisons, even though it may be obvious from the close similarities of their three-dimensional structures. Before examining the *H. influenzae* sequences, therefore, we

assessed the algorithm's capacity to determine homology from sequence by testing it against a set of proteins of known three-dimensional structure.

The evolutionary relationships of protein domains, determined by comparisons of their three-dimensional structures, are described in the Scop database⁵. We took from this database all sequences that have less than 40% identity and carried out an all-against-all comparison using the Smith–Waterman algorithm. The matched sequences were sorted by the ascending order of their *E* values (the expectation of finding such a match by chance), and placed in bins of 20. For each bin, we found the fraction of the 20 for which an evolutionary relationship is also clearly indicated by their three-dimensional structures (see figure). For $E < 10^{-3}$, there are no false positives, whereas at $E > 0.1$ most sequence matches are not supported by structural comparisons. For $E \leq 0.01$, the evolutionary relationships derived from sequence comparisons differ from those derived from structure comparisons by less than 1 case in 100. *E* values between 0 and 0.01, however, detect only 40% of the evolutionary relationships found by structural comparisons (see figure).

Although these values may not be identical for *H. influenzae* sequences, they demonstrate clearly that *E* scores of less than 0.01 reliably detect true relationships, but underestimate the number of evolutionary relationships that actually occur.

We next carried out an all-against-all comparison of the *H. influenzae* sequences, using the Smith–Waterman algorithm, and clustered into families all sequences whose *E* values are less than 0.01. At this level, no relatives are found for 980 *H. influenzae* sequences; the remaining 700 form 208 families (see table). Functions for two-thirds of the *H. influenzae* proteins have been predicted on the basis of their sequence similarities to proteins whose functions are known^{1,6}. Among the remaining third, we find 80 that are related to *H. influenzae* proteins with putative functional assignments and with whom they probably share related functions.

Labadan and Riley⁷ compared 1,264 *E. coli* protein

NUMBER AND SIZE OF PROTEIN FAMILIES IN *H. INFLUENZAE*

<i>n</i> , No. of sequences in a family	No. of families of size <i>n</i>
1	980
2	130
3	46
4	10
5	5
6	5
7	2
8	2
9	1
10	2
13	1
15	1
35	1
42	1
43	1

Total no. of families: 1,188

Total no. of sequences: 1,680

Sequence comparisons were carried out using the SSEARCH implementation of the Smith–Waterman algorithm with In-scaled scores and default parameters⁴. All sequences with *E* values ≤ 0.01 were clustered into families assuming symmetry and transitivity of relationships.

sequences comprising just under a third of the total expected for this bacterium. They estimate that 38–45% of these sequences have arisen from gene duplications. Our examination of the complete set of sequences from the much smaller *H. influenzae* genome found that at least 30% of the proteins have arisen from processes that involve gene duplications. Simple sequence comparisons underestimate the extent to which this occurs and so the true proportions will be significantly greater. It is clear that gene duplications played a major role in the development of even the simplest forms of life.

Steven E. Brenner*

Tim Hubbard†

Alexey Murzin†

Cyrus Chothia*

*MRC Laboratory of Molecular Biology, and

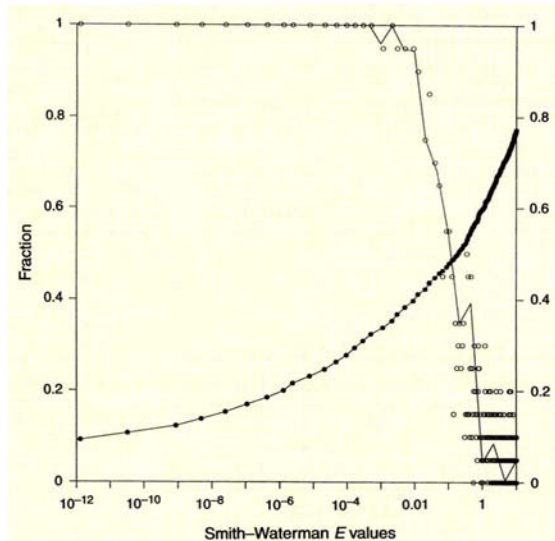
†Cambridge Centre for Protein Engineering,

Hills Road, Cambridge CB2 2QH, UK

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Scientific Correspondence

Scientific Correspondence is intended to provide a forum in which readers may raise points of a scientific character. Priority will be given to letters of fewer than 500 words and five references. Manuscripts can be submitted to London or Washington.



The capacity of the Smith–Waterman algorithm accurately to find protein relationships determined on the basis of three-dimensional structures. We compared the sequences of domains from the Scop database⁵ that have less than 40% sequence identity using the Smith–Waterman algorithm. For groups of 20 sequence comparisons, the fraction for which evolutionary relationships are indicated by similarities in the three-dimensional structure of the domains is shown (○), along with the cumulative fraction of the structurally determined evolutionary relationships detected by the Smith–Waterman algorithm (●). Both fractions are given as a function of increasing values of *E*, the expectation of finding such a match by chance.

Belated appreciations

Martin Bobrow

The History of a Genetic Disease: Duchenne Muscular Dystrophy or Meryon's Disease. By Alan E. H. Emery and Marcia L. H. Emery. *Royal Society of Medicine*, 1, Wimpole Street, London W1M 8AE, UK: 1995. Pp. 248. £20.

DUCHENNE muscular dystrophy is in many ways the perfect model of human monogenic disease. This enjoyable short book brings out many interesting facets. The disorder has a highly characteristic phenotype, but was not fully recognized as a separate clinical entity until the second half of the nineteenth century. It has a classical X-linked recessive pattern of inheritance, only males being affected (with very rare exceptions), whereas unaffected women transmit the disorder. This too was not appreciated until the last quarter of the nineteenth century, more than 25 years after the clinical and pathological features of the disorder were clearly described, which is particularly puzzling because the same familial pattern of haemophilia was known from Talmudic times and formally described in 1803. All these and many other entertaining facts are covered — although with little speculation on explanations for these puzzles — in the early chapters of the Emerys' book.

The driving motive for the authors seems to have been a desire to see more credit given to Edward Meryon, who published clear clinical descriptions of the disorder, with remarkably prescient pathological observations: "... the sarcolemma or tunic of the elementary fibre was broken down and destroyed". That was in 1852, nearly ten years before the early publications of Guillaume Benjamin Amand Duchenne, the French neurologist

whose name has been associated with the disease ever since. Why was Meryon's work buried? The hypothesis here is that Meryon was a retiring person, who published his important observations in one paper, with a short book chapter 15 years later. The energetic Duchenne published frequently, lectured often and generally made sure people knew of his work. Again, the disease provides illustrations more than a century old of issues still current today. That the Emerys think a lot of Meryon is evident from the table of contents. From 205 pages of text, he gets 30 pages to Duchenne's 19, and 24 for the whole of modern molecular genetics.

This fascination with Meryon, Duchenne and their predecessors and contemporaries infuses the first half of the book, making delightful reading. Discursive background material, to set the issues in proper social and historical context, adds to the pleasure. Where else could I have learned that Duchenne designed a version of the modern muscle biopsy needle, which he kept from rusting by storing it in alcohol, thus accidentally avoiding problems of sepsis, about the causation of which he could have known nothing? The distinguished anatomist and surgeon Sir Charles Bell owned a building that subsequently became the Lyric Theatre in London's West End. It was a school of anatomy then, "the present stage door formerly being the entrance for 'subjects for dissection'".

The authors give appropriate and welcome credit to Newton Morton, who introduced discriminant and segregation analyses into modern human genetics as part of a large population study of Duchenne muscular dystrophy. Tony Murphy used the disease as the basis for developing Bayesian risk-analysis procedures. These contributions have long since permeated much of clinical genetics.

Muscular dystrophy also provided the basics for a very different development. The Muscular Dystrophy Association was formed in the United States in

1950, and the Muscular Dystrophy Group in the United Kingdom in 1959. They were forerunners of the enormously important movement for patient and family advocacy groups, to raise funds for care and research and to maintain political pressure on behalf of those suffering from relatively uncommon diseases.

The latter half of the book lacks, to my mind, the same drive and focus. Various other muscular dystrophies are described at a level of clinical detail that strays perilously close to a basic medical text rather



Duchenne: energetic self-publicist.

than an historical account. The massive fundamental advances of the past decade are superficially described, liberally laced with thumbnail sketches of a selection of the important contributors. These are constructed with sufficient similarity to make reading them somewhat like leafing through a pile of applicants for an appointments committee — although it would be a very lucky committee to have drawn quite this group of applicants.

On the opening page, the authors write that "... the history of the disease is unique because it encapsulates so many developments in medical science over the last 150 years". I rather suspect that Duchenne muscular dystrophy is not all that unusual, and that the real interest of the story is as an exemplary model for many other biomedical advances. As such, and because of the strong overt commitment to Edward Meryon, at least the first half of the book should appeal to a wider audience than just those interested in muscular dystrophy. □

Martin Bobrow is in the Department of Medical Genetics, University of Cambridge, Cambridge CB2 2QQ, UK.



Meryon: astute but retiring observer.

Postmortem of a cometary suicide

Don Yeomans

Impact Jupiter: The Crash of Comet Shoemaker-Levy 9. By David H. Levy. *Plenum*: 1995. Pp. 290. \$25.95, £20.76.

The Great Comet Crash: The Collision of Comet Shoemaker-Levy 9 and Jupiter. Edited by John R. Spencer and Jacqueline Mitton. *Cambridge University Press*: 1995. Pp. 118. £16.95, \$24.95.

RECENT estimates suggest that a comet is captured by, and collides with, Jupiter once every several thousand years. What extraordinary luck that it happened on our watch in July 1994. It seems likely that comet Shoemaker-Levy 9 was captured by Jupiter around 1929 and spent several years in chaotic orbits about the

president of the United States. Designed for the general reader, Levy's book is a well-illustrated account of the comet's discovery, the events leading up to the collision events and a summary of the scientific consensus resulting from the impacts themselves. In characteristically lucid and entertaining style, Levy presents the reader with his reminiscences and anecdotes, interesting historical background items and an overall outline of what was expected and what actually transpired.

Many in the scientific community predicted that the impacts would fizzle; and, in the unlikely event that the various impact sites would be visible on the surface of Jupiter, that they would be white and observable only with the largest telescopes. The actual impacts were far from the expected fizzle. Although they were most dramatic at infrared wavelengths rather than in the visible region, the energies of the fragment collisions

dwarfed any terrestrial nuclear explosions and the resulting black scars on Jupiter were easily observable with modest amateur telescopes. Each impact happened just behind the visible limb of Jupiter but the camera system on board the Galileo spacecraft, which was on its way to a Jupiter encounter in December 1995, was in a position to see several of the hits directly.

For a more in-depth treatment of the scientific issues surrounding the collision of Shoemaker-Levy 9 with Jupiter, readers should turn to the work edited by John Spencer and Jacqueline Mitton. Each chapter is written by an expert in the field, and although there is some

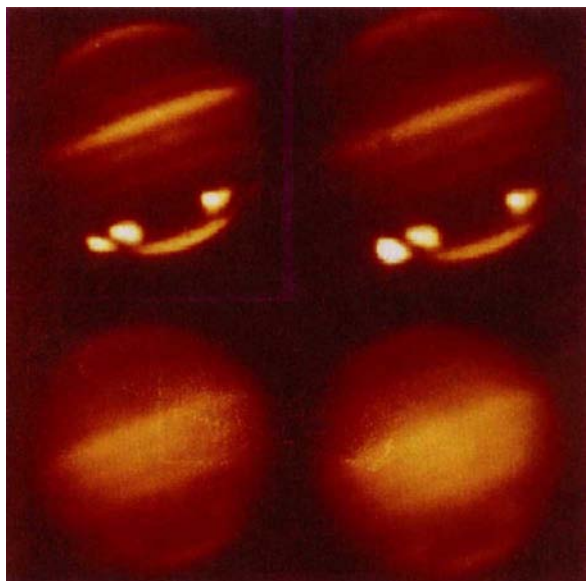
overlap among the chapters, the book presents a comprehensive review of the discovery observations, as well as the pre-crash observing plans and post-crash results. The volume is well written and handsomely illustrated with images from ground-based telescopes, the Galileo spacecraft and the Hubble Space Telescope (which had recently had its optical system repaired).

There are readable summaries of what was learned about Jupiter's atmosphere and the comet itself. The existing models of Jupiter's atmosphere seem fairly accurate but the current model of the comet's nucleus has been called into question. Rather than its being a single body of ice and rock that the low-resolution images of comet Halley seemed to reveal, the tidal break-up of Shoemaker-Levy 9

suggests that this comet, and perhaps others as well, was an extremely fragile body with sizeable kilometre- or sub-kilometre-sized fragments of dust, rock and ice. At the time of the break-up in 1992, all of the fragments were bound to one another with little more than self-gravity. Perhaps this comet spent its first 4.5 billion years since the origin of the Solar System gradually losing the ice that initially glued the various fragments together. Toward the end of its lifetime, the suicidal comet was reduced to a feeble collection of fragments that had the misfortune to plunge into Jupiter's atmosphere.

In Levy's book, Gene Shoemaker is quoted as saying: "[N]ow what are the odds to have such a rare event happen in the decade that all the new infrared detectors became available, as the Galileo spacecraft was in position to see the hits directly, and only six months after the Hubble [Space Telescope] was fully operational and before the expected cutbacks [in NASA's budget] make the money run out? Folks, we had a bloody miracle." Indeed, and these two books make an excellent choice to document that miracle. □

Don Yeomans is at the Jet Propulsion Laboratory, California Institute of Technology, Pasadena, California 91109, USA.



SPL/Clark, Pryor et al., McDonald Observatory

Infrared images of the collision of comet Shoemaker-Levy 9 with Jupiter, made at the McDonald Observatory, Texas.

planet. In July 1992, the comet passed very close to Jupiter's surface, whereupon the planet's tidal forces split the comet into several fragments. After one final two-year orbit, all the comet's fragments collided with Jupiter during the week of 16–22 July 1994. Fortunately, the comet was discovered in March 1993 by Gene and Carolyn Shoemaker and David Levy, allowing the astronomical community 16 months to prepare for the impact events.

David Levy has provided his personal account of the discovery of this extraordinary comet and the events and circumstances that swept him and the Shoemakers into the media limelight for several months. Their frenzied activities included several press conferences and meetings with the president and vice-

New in paperback

Descartes' Error: Emotion, Reason, and the Human Brain by Antonio R. Damasio. Avon, \$12.50.

"Damasio's arguments are ingenious and wide ranging. . . His thoughtful and modest exposition should be taken seriously. Apart from illuminating the function of the frontal lobes, he has proposed a new physiological mechanism that is likely to be much investigated over the next few years. It is no mean feat to say something original and intelligible about emotion". Stuart Sutherland, *Nature* **372**, 387 (1994).

Modeling Nature: Episodes in the History of Population Ecology by Sharon E. Kingsland. University of Chicago Press, \$16.95, £13.50. Now in a second edition, with a new afterword that looks at the rise of "the new natural history" and debates about ecology's future as a large-scale scientific enterprise. "Unfolds like a good detective story, and is a delight to read. . . Beautifully written, well-crafted account of a complex subject". John H. Lawton, *Nature* **322**, 603 (1986).

Foundations of Neuroscience by Marcus Jacobson. Plenum, \$47.40, \$31.60. "It does an important service by enquiring deeply into the origins and philosophical implications of the underlying ideas of neuroscience". Mitchell Glickstein, *Nature* **369**, 369 (1994).

Dealing the race deck

Jonathan Marks

The Race Gallery: The Return of Racial Science. By Marek Kohn. Cape: 1995. Pp. 322. £17.99.

ONE of the most astonishing features of contemporary discussions of race is the fact that anthropology, the science that deals with human biological and cultural variation, has managed to be marginalized. From the public furor over *The Bell Curve* to the design of the Human Genome Diversity Project, anthropology has become a science of which folk knowledge is widely perceived to suffice.

Yet it is precisely where folk knowledge is most deeply entrenched that science is the most revolutionary and the most threatening. In the sixteenth century, astronomy told us that the Earth rotates, despite the fact that anyone can plainly see the Sun rise and set. In the twentieth century, anthropology has a comparably revolutionary message: the human species does not come packaged into a small number of reasonably discrete kinds of people. Rather, it forms a series of gradients, arbitrary divisions of which create disjunctions and associations among populations. To the extent that animosities exist between populations, these are motivated by social, not biological, differences — for the most similar peoples are often the most hostile. This tells us a bit about our desire to make sense of the world by classifying it, but far more about the ever-present danger of mistaking culturally constructed patterns for those in nature.

The anthropological message has become transformed over the past generation into a vacuous tenet of political correctness. Alongside it is a folk caricature of anthropological idealism: that because races are culturally constructed, everyone is the same as everyone else.

If anything, of course, the very opposite is true — everyone is different. At issue is the general pattern that structures the natural variation in our species. Across the aboriginal landscape, people are similar to those nearby and different from those far away. And this no more tells us that there are three different kinds of humans than it tells us that there are six, or twelve, or thirty.

So the conclusion to *The Race Gallery*

is something of a *non sequitur* in relation to the relevant science: "[T]he time has come", Marek Kohn says after some 284 pages, "to explore how biological variation and social construction are related. Dealing with difference may be easier said than done. But denial no longer appears to be an option." So racism is bad, but people are different. Kohn seems to be criticizing an anthropologist of his own construction, intoxicated with fluffy-headed notions of the unity of humankind and denying that anyone is different from anyone else.

Lest I stray too far afield here, it must



From *Principles of Visual Anthropology* edited by Paul Hockings, second edition. Mouton de Gruyter, \$59.95 (hbk), \$29.95 (pbk).

be said that there is much to admire in this book. Its heart is in the right place. After all, this is a political-intellectual arena in which there are always rewards for those heartless enough to invoke nature as a predominantly causal factor in the distribution of human misery. And Kohn is not among them. But the subject of the book is ostensibly biological anthropology, and its bibliographic notes contain extraordinarily few references to that literature. Apparently folk knowledge suffices. That is, indeed, the nub of the problem.

Kohn's chapter on the Human Genome Diversity Project is perhaps the book's most astute contribution, observing that it "speaks the language of non-racism and anti-racism... [but] is

wont to lapse into older dialects when it addresses lay audiences". Yet even here, Kohn misses the bull's-eye. All of the project's problems stem from the simple fact that it began as an anthropological programme designed by molecular population geneticists, armed with only a folk knowledge of anthropology. Thus, its archaisms surface not simply in lay presentations. In *Proceedings of the National Academy of Sciences* we can read that the European gene pool was formed of 65 per cent Chinese and 35 per cent African genes, as if it were a veritable racial martini. In a scientific tome, a high-tech map of the world's genetic structure has four colour-coded "ethnic regions" imposed on it — Caucasoids, Mongoloids, Australians and Africans — as if the problem were semantic rather than conceptual.

Kohn indeed offers no substantial critical analysis of the field of genetics, relevantly and effectively accomplished by the sociologist Dorothy Nelkin and the historian Susan Lindee in their recent book *The DNA Mystique* (for a review, see *Nature* 113, 377; 1995). Genetics was, after all, the field that made racial inferiority respectably scientific in the 1920s. Every quotation Kohn adduces from 1940s' Germany is an echo of mainstream American genetics textbooks of the 1920s.

In failing to tackle the history or meaning of genetics critically, Kohn is able to repeat the topsy-turvy idea that the arguments against race made by Ashley Montagu after the Second World War were nice, but supported only later by genetic discoveries. In fact, the paradigmatic genetic cline, the ABO blood group, was well understood by the mid-1920s. But it was invariably interpreted within the race concept, as were all other genetic data throughout the early 1960s. Kohn observes in passing that the original students of ABO variation "suggested a double origin of the human species, the B race arising in India and A race in Northern or Central Europe. Their contemporaries were frustratingly slow to accept their ideas." As well they should have been: genetic data *per se* actually had little to do with establishing the modern view; they were simply reinterpreted within it.

The Race Gallery admirably engages contemporary racist teaching in the personae of Richard Lynn, J. Philippe Rushton, Richard J. Herrnstein and Charles Murray, and others — mostly heterodox psychologists. The reified, ostensibly some innate scalar brain force measured by the most obsessive enthusi-



IN May 1915, a German submarine sank the Cunard liner *Lusitania*. But rumours of conspiracies and cover-ups surround the event. Why did the ship sink so quickly? Why was there a second explosion? Had the liner been carrying munitions for the British? Did Churchill allow the sinking in the hope that it would draw the United States into the war? In *Exploring the Lusitania*, Robert D. Ballard, the discoverer of the *Titanic* and the *Bismark*, gives some answers, based on the findings of an expedition he led in 1993 to the wreck of the *Lusitania*. His underwater photographs accompany many archival photographs, paintings and diagrams. Madison/Weidenfeld and Nicolson, \$20.

asts of IQ, comes in for effective treatment. The book also deals quite competently with the folk ideas of racial superiority in sports. Its centrepiece involves the antiquated local anthropology validating the subjugation of Eastern European Gypsies, a tragic and sobering read.

The book is therefore principally about current events rather than being a weighty scientific discussion of underlying issues. Nevertheless, for all its shortcomings as a scholarly analysis, this is a useful book, not to be tossed away. It strives to be on the side of the angels, and on the side of science (although I am not entirely convinced the author fully understands the basis for this). In style it is often glib without plummeting into condescension. The author appreciates the gravity of the topic he has chosen and clearly wishes his book to do some good in the world. That is admirable, and I hope and expect that his wish will be fulfilled.

Ultimately, though, he is in over his head. There really are some thoughtless, disingenuous or downright evil scientists out there, establishing their reputations on the backs of the downtrodden and voiceless. That is bad all around — bad for society and bad for science. Effectively debunking them requires a hierarchical vision, involving not only scientific critiques of methodology and inference but also meta-scientific critiques of just what science can and cannot say about certain things, the role of science as validation (and the attendant responsibilities

of scientists) and the very nature of scholarship and academic professionalism. Otherwise we are left simply with people who do think there are basic divisions of the human species with unequal worth, and people who don't — in other words, a legitimate-seeming scientific impasse, which ultimately works to the detriment of both science and social progress. The only bona fide student of human biological variation we meet in this book is Fatimah Jackson, who is curiously represented as an Afrocentric antithesis of Rushton — in this way simultaneously discrediting the mainstream Jackson and legitimizing the atavistic Rushton.

With a firmer grounding in the relevant scientific literature, *The Race Gallery* could have broken down the problem of racial science into an answerable series of smaller questions.

First, is the human species divisible into a small number of natural groups? No. This was known to Buffon and Blumenbach and summarized epigrammatically by Frank Livingstone in 1962 (as quoted by Kohn): "There are no races; there are only clines".

Second, is it legitimate to compare populations derived from diverse regions? Yes. Primary research in journals such as *American Journal of Physical Anthropology*, *Human Biology*, *Annals of Human Biology* and *American Journal of Human Biology* has been doing this for decades. (This literature is not cited by Kohn.)

Third, if they are found to differ, is the

difference biologically based? Maybe. Different facial features of a Swede and a Yoruba may be biologically based, but the different languages they speak are surely not. A century of acculturation studies shows that people's minds and bodies can change radically within a generation after migration. A consistent observation of difference is therefore not a reliable indicator of a biological basis for that difference. A specific observed difference may be constitutionally rooted, but requires a higher standard of validation than just consistency of observation to establish it.

Finally, do populations have different intellectual abilities? Probably not. The reason is simply that ability is a folk concept and not amenable to scientific analysis. Because a test is a performance, and ability is only one component of a performance, it follows that performance is not a reliable estimator of ability. A good performance is a demonstration of ability; a bad performance may not at all be a demonstration of the lack of ability. So the fundamental falsehood is the claim that we can say anything scientific at all about individual ability or potential — much less those of groups. History does tell us, however, that populations derided for their abilities in one era are often quite competent in another.

Without a scientific approach to human potential, we are left with two poles of social action: we can try to identify and cultivate diverse talents as widely as possible (by investing our resources in education, day care and children's enrichment programmes); or we can condemn large groups of people on the basis of their lack of performance, denying both the right of the subjects to be judged as individuals and the possibility of society benefiting from the talents with which they are endowed. Neither alternative is particularly scientific; but the first is at least humanitarian. □

Jonathan Marks is in the Department of Anthropology, Yale University, PO Box 208277, New Haven, Connecticut 06520-8277, USA.

Children's Books

Next week's issue contains *Nature's Children's Books* supplement, in which reviewers — young and old alike — will look at some of the science books and software for children recently published in the United Kingdom and the United States. Subjects include dinosaurs, natural history, computers, fire, energy, the weather, genetics and drugs. And Frances Balkwill, author of several award-winning children's books, including *Cells Are Us* and *The Egg and Sperm Race*, offers her thoughts on the business of writing successful books for youngsters.

Bifurcations of the Atlantic thermohaline circulation in response to changes in the hydrological cycle

Stefan Rahmstorf

Institut für Meereskunde, Düsternbrooker Weg 20, 24105 Kiel, Germany

The sensitivity of the North Atlantic thermohaline circulation to the input of fresh water is studied using a global ocean circulation model coupled to a simplified model atmosphere. Owing to the nonlinearity of the system, moderate changes in freshwater input can induce transitions between different equilibrium states, leading to substantial changes in regional climate. As even local changes in freshwater flux are capable of triggering convective instability, quite small perturbations to the present hydrological cycle may lead to temperature changes of several degrees on timescales of only a few years.

THE North Atlantic Ocean today carries $(1.2 \pm 0.2) \times 10^{15}$ W of heat northwards^{1,2}. Most of this heat transport is due to the vertical overturning cell associated with North Atlantic Deep Water (NADW) formation³; warm surface water flows northwards, sinks, and flows southwards as cold deep water. The volume transport of this 'conveyor belt' is about 17 ± 4 Sv (ref. 3; 1 sverdrup = 10^6 m³ s⁻¹). This heating system makes the northern North Atlantic about 4 °C warmer than corresponding latitudes in the Pacific⁴ and is responsible for the mild climate of Western Europe. Variations in NADW circulation therefore have the potential to cause significant climate change in the North Atlantic region.

Previous modelling studies have suggested that the NADW circulation is a nonlinear system which is highly sensitive to changes in freshwater forcing; it may collapse if a certain threshold is exceeded⁵⁻⁹ and can show hysteresis behaviour^{10,11}. There is mounting palaeoclimate evidence, particularly from sediment cores, showing that past climate shifts were associated with changes in NADW flow¹²⁻¹⁵. Recent coupled ocean-atmosphere model experiments investigating the effect of anthropogenic greenhouse gases on climate find that global warming would probably lead to reduced NADW formation^{16,17}.

Given the possibility of an anthropogenically triggered transition in NADW circulation, a systematic study of its sensitivity is urgently needed. Excessive computation costs make this not yet feasible using fully coupled ocean-atmosphere models. Here, a study of circulation transitions made with a global ocean circulation model coupled to a greatly simplified atmospheric model is presented. It is found that NADW circulation winds down when a bifurcation (first suggested by Stommel⁵) is passed, which the model predicts to occur when an additional freshwater input of less than 0.06 Sv is introduced into the catchment area of the North Atlantic. Convective instability can trigger a local shutdown of deep convection for even smaller regional changes in freshwater forcing, leading to rapid regional sea surface temperature (SST) changes within a few years. A transition to oscillatory behaviour³⁸ (a Hopf bifurcation) was also discovered, leading to a parameter range with self-sustained interdecadal oscillations.

Model and experiment design

The ocean model used in this study is the general circulation model (GCM) developed at the Geophysical Fluid Dynamics Laboratory (GFDL) in Princeton¹⁸, in a coarse-resolution global configuration (Fig. 1a) with twelve vertical levels, similar to the models used in refs 17, 19 and 39. The model was driven by observed annual mean wind stress²⁰. The surface flux of fresh

water was derived from a spin-up experiment and prescribed as a fixed flux thereafter; this flux field is illustrated in ref. 21, where details of the model spin-up are also described. Regional integrals of the flux are shown in Fig. 1b; perturbations added to the freshwater flux for the sensitivity experiments are described below.

For thermal forcing, neither prescribing a fixed heat flux nor restoring the SST to prescribed values provides a realistic feedback response to large-scale ocean circulation changes²². Therefore, a simple atmospheric energy balance was used, which damps SST anomalies by horizontal diffusion of heat in the atmosphere and by longwave radiation. This approach is discussed in detail in refs 21 and 22. It reproduces the correct SST contrast between North Pacific and North Atlantic oceans in the model equilibrium as a result of oceanic heat transport, without prescribing it in the forcing. The damping of SST anomalies depends on their spatial scale; small anomalies are removed rapidly by atmospheric transport, and the largest scales damped only by longwave radiation to space. Scale selectivity is crucial for successfully modelling local heat loss in small convection regions at the same time as the large-scale response to oceanic heat transport.

With this surface forcing, the model reaches a steady equilibrium state after an integration time of ~5,000 years. This is used as a starting point for the experiments described below. In this state, the Atlantic thermohaline circulation has the familiar two-storey structure with a NADW overturning cell of 20 Sv stacked above an opposite turning cell of Antarctic Bottom Water of 7 Sv; the Pacific and Indian oceans derive their entire deep water from an inflow of 11 Sv from the south.

In the experiments reported here, slowly varying freshwater flux perturbations were added in different ocean regions (Fig. 1a). The perturbations were increased or decreased linearly in time, in order to trace the hysteresis response of the ocean circulation. The method was inspired by a study by Mikolajewicz and Maier-Reimer¹¹, who investigated the effect of thermal restoring boundary conditions on freshwater discharge events of 1,000–2,000 years duration. The aim of our study is different; we attempt to trace the equilibrium response of the NADW circulation to freshwater forcing changes, that is, to construct a bifurcation map and to identify critical transitions. Consequently, the forcing was varied at a much slower rate (up to 230 times slower than described in ref. 11). Freshwater flux is used as control variable for the experiments and is therefore specified. In reality there is a feedback of sea surface temperature on the freshwater flux²³, but recent work suggests this is only weak²⁴.

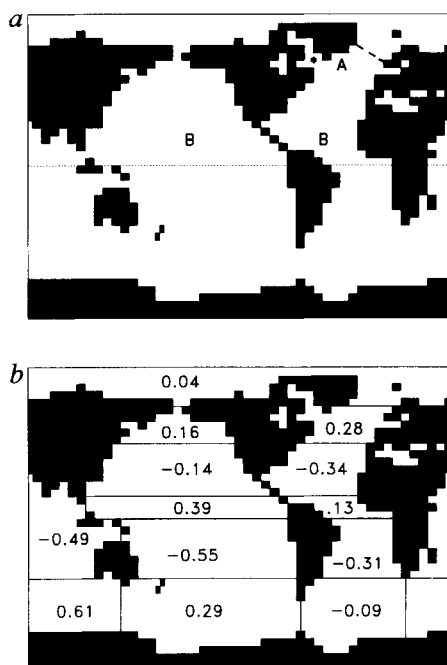


FIG. 1 *a*, Location map for the model experiments. The shaded areas A and B mark regions where freshwater forcing was varied; for region B the change was of opposite sign in the Atlantic and the Pacific. The Labrador Sea is marked with an asterisk, and the Greenland-Iceland-Scotland ridge separating the Atlantic from the Arctic is indicated as a dashed line. *b*, Regional integrals of net freshwater forcing in sverdrup ($1 \text{ Sv} = 10^6 \text{ m}^3 \text{ s}^{-1}$). Positive values indicate net precipitation and occur in the intertropical convergence zone and in high latitudes; the subtropical gyre regions are characterized by net evaporation. The fluxes were obtained by forcing the ocean model towards observed surface salinity⁴, and thus include the effect of river runoff. Note that there is no net volume transport and negligible net freshwater transport through Bering Strait in this model, so that oceanic transport across a given latitude in the Atlantic can be obtained by summing the surface fluxes from the Arctic; at the latitude of South Africa, the ocean circulation exports salt equivalent to a freshwater inflow of 0.2 Sv.

Conveyor hysteresis

Figure 2 shows the NADW circulation (defined as the maximum of the meridional mass transport in the Atlantic, excluding the near-surface wind-driven layers) as a function of the freshwater perturbation for a number of experiments. In Fig. 2, top panel, fresh water was added to a region south of Greenland (labelled A on Fig. 1*a*), with the inflow increasing or decreasing by 0.05 Sv per 1,000 years; Fig. 2, bottom panel, shows experiments where fresh water was taken from the tropical Pacific and added to the tropical Atlantic (in the regions labelled B on Fig. 1*a*). Figure 2 bottom also includes two additional experiments where the forcing was changed at the slower rates of 0.006 and 0.02 Sv per 1,000 years. The initial state is labelled *a*.

The hysteresis curves show a decline in NADW overturning with increasing freshwater input into the North Atlantic. Beyond a certain value, overturning is essentially zero (the small remaining background transport is due to the fact that the meridional stream function never becomes negative everywhere, even in the absence of NADW formation). For an intermediate forcing range which includes present-day climate, NADW overturning can be either 'on' or 'off' (as in equilibrium *f*), depending only on initial conditions.

The inflow of Antarctic Bottom Water into the Atlantic decreases slightly as NADW flow weakens in the model. In the Pacific, the bottom water circulation also weakens, and after the breakdown of NADW flow an overturning cell of 10 Sv starts between 30° N and 60° N. This North Pacific cell (not shown) reaches 1,500 m depth and recirculates within the Northern

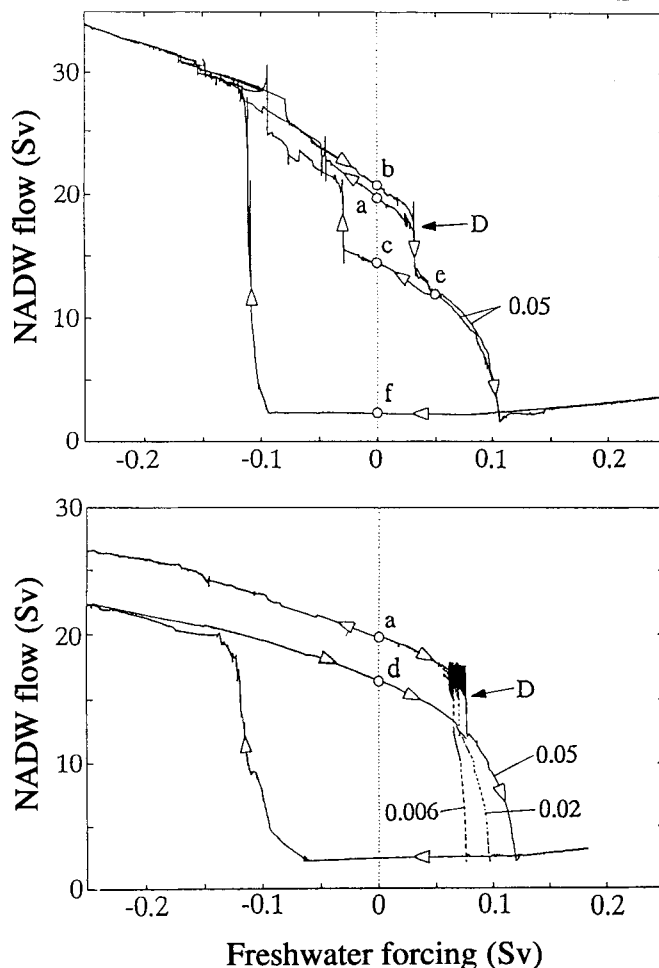


FIG. 2 Hysteresis response of the North Atlantic overturning circulation to a slowly changing freshwater forcing in the high latitudes (top panel) and the tropics (bottom panel; see Fig. 1 for exact locations). (NADW, North Atlantic Deep Water). Open circles mark true model equilibria obtained with constant freshwater forcing; the equilibrium marked *a* is the initial state at the start of the experiment. Arrows mark the direction in which the curve is traced. After one hysteresis loop, starting from *a* towards the right, the model arrives at equilibrium *b*. From there the right half of the hysteresis curve was traced a second time. Point *c* was reached by reversing at point *e*. Point *f* is an equilibrium with no NADW formation, under present-day forcing. The discontinuity *D* is the point where Labrador Sea convection ceases. In the lower panel, the right half of the hysteresis curve was traced at three different rates; the rate of freshwater forcing change is labelled in units of $10^{-3} \text{ Sv yr}^{-1}$.

Hemisphere with no outflow to the south, resembling the hemispheric circulation envisaged in Stommel's⁵ original box model. It shows a clear hysteresis response even if the forcing changes only in the North Atlantic, constituting an interesting oceanic teleconnection between Atlantic and Pacific oceans.

Saddle-node bifurcation

The general shape of the hysteresis curves and their bimodal structure can be understood with the help of Stommel's⁵ classical box model of the thermohaline circulation (which can be generalized to allow cross-hemispheric flow). Figure 3 shows the equilibrium solutions of this simple model as a function of freshwater flux. The positive branch corresponds to the overturning of NADW, while the negative flow branch corresponds to a reverse flow. The branch shown dashed is unconditionally unstable, and separates the basins of attraction of the two stable branches. Point *S* is a turning point, or saddle-node bifurcation, beyond which no stable positive solution exists. The thin lines were obtained by time integration with slowly increasing fresh-

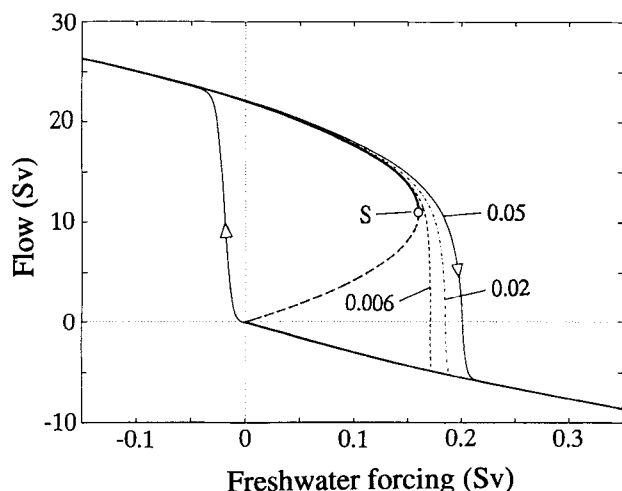


FIG. 3 Equilibrium flow and hysteresis response of Stommel's box model. Heavy curves show the analytical equilibrium solution; the dashed branch is unstable. S is the location of the saddle-node bifurcation. Thin lines are the result of a time integration with a freshwater forcing cycle identical to that used in the GCM experiments shown in Fig. 2. Note that freshwater forcing is given in absolute values for the box model, whereas for the GCM it is relative to the present-day climate.

water flux, as in the GCM experiments shown in Fig. 2. They show that the equilibrium branch can be tracked closely by a slow forcing increase—except near the bifurcation point, where the model response becomes infinitely sluggish.

A crucial question is where Stommel's bifurcation is located in the real world, and, as an approximation to this, in the GCM. In other words, what is the critical change in hydrological forcing which would trigger the shutdown of the present NADW conveyor belt circulation? From the box-model analogy, we conclude that the hysteresis experiments with the GCM can only provide an upper limit of this value. Figure 2, bottom panel, shows three GCM runs with different rates of forcing change (as in Fig. 3) and gives an upper limit of 0.075 Sv for the critical flux. To test for equilibrium, in a number of further experiments freshwater forcing was held constant after certain values had been reached. For the high-latitude perturbation (Fig. 2, top), the model was still stable and the hysteresis curve close to equilibrium at a flux of 0.05 Sv, but at 0.06 Sv it was beyond the bifurcation and wound down. Similarly, for the low-latitude perturbation the bifurcation was found to be between 0.056 and 0.062 Sv. The corresponding critical rate of NADW overturning is close to 12 Sv; smaller rates of NADW formation cannot apparently be sustained by the model.

It is remarkable that the critical amount of freshwater input is essentially the same, whether added in the high-latitude convection region or in the low latitudes. This is because the mechanism underlying Stommel's bifurcation is a large-scale advective mechanism. It does not much matter where the fresh water is added, as long as it passes through the NADW loop before leaving the Atlantic, that is, it is added to the "NADW catchment area". This catchment area will not have a sharp boundary in a turbulent ocean; for example, fresh water added to the South Atlantic can be expected partly to flow north in the conveyor and partly to exit to the Southern Ocean through wind-driven circulation. The catchment area also depends on the circulation itself.

It is difficult to assess which changes in atmospheric circulation would be required to increase the freshwater transport into the NADW catchment area by 0.06 Sv, but Fig. 1b indicates that the change would only be a fraction of the climatological flux. In the coupled experiment of Manabe and Stouffer^{17,25} for a quadrupling of the atmospheric level of CO₂, NADW formation is completely extinguished by a net precipitation increase

in the North Atlantic, while sea-ice melting makes a negligible contribution. If a freshwater flux of 0.06 Sv were to be obtained only by melting sea ice, the entire Arctic sea-ice volume ($2-3 \times 10^{13} \text{ m}^3$) would need to melt during a period of 10–15 years, a time span too short to cause an advective spin-down of the circulation. The timescale of the shutdown is determined by the advection of salt in the overturning circulation and is typically several hundred years; the more the critical flux is exceeded, the faster the spin-down. Sea-ice melting is, therefore, not a viable mechanism for causing an advective spin-down, but could lead to convective transitions as discussed in the next section. For comparison, meltwater inflow from the disintegration of continental ice sheets at the end of the last glacial peaked at 0.44 Sv and lasted many centuries²⁶.

Convective instability

Although the overall shape of the hysteresis curves can be explained by Stommel's box model, there are also interesting discontinuities, most notably the one marked D in Fig. 2. When we start from point e on the equilibrium curve and move to the left, a new equilibrium branch is found with a lesser NADW overturning rate. And when a full hysteresis cycle is completed, we arrive at not the initial state a, but a different equilibrium state (b or d respectively). We are thus finding multiple equilibrium states with different NADW formation rates under the same forcing.

Similar multiple equilibria have previously been found and analysed in idealized models^{27–29}; they are linked to different stable convection patterns and are caused by a positive feedback mechanism which can make convection self-sustaining once it has been established at a certain point. The convection patterns for several equilibria marked in Fig. 2 are shown in Fig. 4. The crucial difference between model states with ~ 20 Sv overturning and those with only ~ 16 Sv overturning is the absence of Labrador Sea convection in the latter. Time series of convection depth at single points (not shown) confirm that at the discontinuity D Labrador convection shuts down. Other small jumps in the circulation time series can generally be attributed to a change in convection somewhere in the model. Associated circulation changes appear almost instantaneous, as adjustment to convection changes occurs through a mechanism of wave propagation^{30,31} which takes only a few years to complete. Convective instability is therefore a mechanism that could lead to rapid climate shifts such as the Younger Dryas event²⁹.

Convective instability does not depend on the large-scale freshwater budget but on local flux. This is why the shutdown of Labrador convection occurs for a much smaller freshwater input for the high-latitude perturbation than for the low-latitude perturbation (compare Fig 2 top and bottom). Further experiments showed that a perturbation of 0.015 Sv is enough to cause the shutdown if targeted directly at the Labrador Sea. This demonstrates that rather small changes in freshwater forcing may lead to regional shutdown of convection. In the real world and in fully coupled models, the distinction between similar equilibria (such as a and b) may be blurred by atmospheric variability, but qualitatively different convection patterns (such as a and c) should represent distinct climate states (see the discussion in Rahmstorf²¹). Sediment-core results¹⁵ have linked past climate change to a regional shift in NADW convection, from the Norwegian Sea to a location south of Iceland.

Interdecadal oscillations

A further interesting feature of the hysteresis curves is the occurrence of interdecadal oscillations in certain parameter ranges. The most spectacular oscillation arises in Fig. 2, bottom panel, just before the Labrador discontinuity D. Figure 5 shows a close-up view of the relevant section of the curve; it reveals that a Hopf bifurcation (H) leads to a transition from a stable model solution to a limit cycle with a period of 22 years. This period is independent of the rate at which freshwater input is increased.

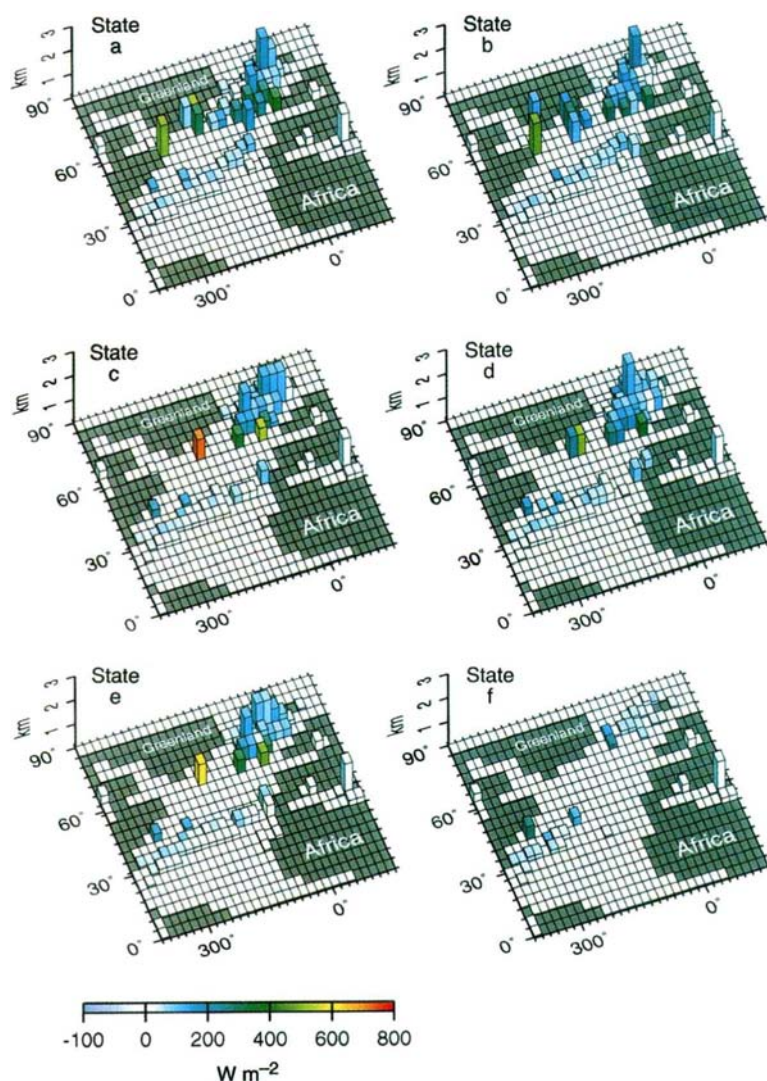


FIG. 4 Convection patterns in the North Atlantic for six equilibrium solutions (states a–f) of the ocean model as labelled in Fig. 2. The height of the columns shows convection depth from the surface, and colour indicates the amount of heat (W m^{-2}) brought to the surface by convection. Convection is the main process by which the heat brought to the north in the conveyor circulation is released from the water column. Note that states a and b have convection in the Labrador Sea, whereas in c–e NADW is formed only east of Greenland. State f is a 'southern sinking' state without NADW formation.

The overturning rate varies by as much as 3 Sv, and the average temperature of the high-latitude North Atlantic by 0.2°C . The temperature maximum lags behind the overturning maximum by about 3 years. As one would expect from the short period, this oscillation is local to the North Atlantic; it is also deep—the flow oscillates in a region between 20°N and 60°N over the whole depth range. Surprisingly, Labrador Sea convection is not affected; convection depths remain completely stationary throughout the oscillation. The sea surface temperature pattern associated with the oscillation is shown in Fig. 6a. With its maximum near Newfoundland, it resembles the observed interdecadal variability pattern shown by Kushnir³², except that it lacks the second maximum found in the data near the sea-ice margin north of Iceland. This second maximum may be linked to sea-ice variations; no ice model is included in the present study. A much weaker oscillation with a period of 16 years arises in the high-latitude experiment (Fig. 2, top), again just before the Labrador convection shuts down. Similar interdecadal SST oscillations centred on the Labrador region have been found in a number of studies with different models^{33–35}.

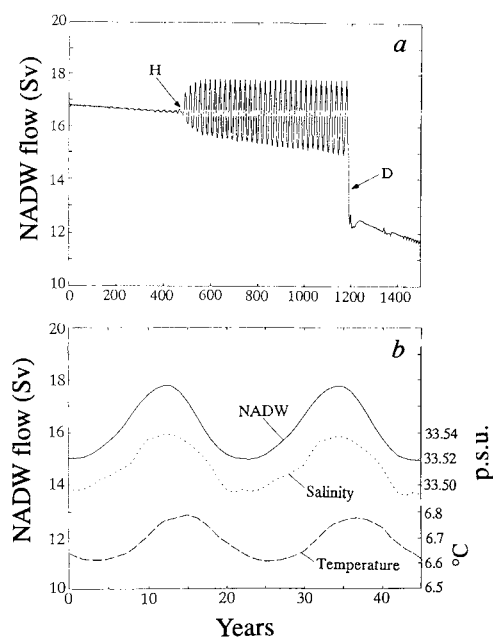


FIG. 5 a, Close-up view of the oscillations visible in Fig. 2, bottom panel, just before the shut-down point D of Labrador convection. The time axis is labelled in years with an arbitrary origin. At point H, the model passes a Hopf bifurcation, where the stable equilibrium state gives way to a limit cycle with a period of 22 years. Note that the amplitude initially increases as the square root of the control parameter (freshwater flux, which in this case is proportional to time); a characteristic feature of Hopf bifurcations³⁸. b, Two periods of the oscillation at higher time resolution. Also shown are surface salinity and temperature, averaged over the Atlantic north of 50°N .

Consequences for climate

Given the large heat transport of the NADW conveyor belt, it is natural to ask what the bifurcations described above mean for Atlantic surface temperatures. Because of the simple atmospheric heat budget employed, the model has a limited capacity to predict SST changes resulting from circulation changes. The equilibrium with the strongest overturning (state b), also has the warmest high-latitude SST. Figure 6 shows SST differences of other model states compared to initial state a, as well as the SST change over one of the oscillation cycles discussed above (Fig. 6a).

The interdecadal oscillation has a range of 0.7°C near Newfoundland and decreases to under 0.2°C near the European coast. The difference between normal and weak NADW circulation equilibria (Fig. 6b) is larger; it reaches a maximum of 2.5°C near Newfoundland. The cooling predicted for a total shutdown of NADW circulation (Fig. 6c) reaches a maximum of 6°C near Greenland, with widespread cooling of over 4°C across the Atlantic. Note that a fully coupled experiment would probably show an amplified temperature response near ice margins due to the ice-albedo feedback (not included in this model). The cooling effect would also extend further north in a model that produces more NADW north of the Greenland–Scotland ridge.

Overall features of the bifurcation map

The experiments discussed in this Article provide a map of the equilibrium states and bifurcation points of the Atlantic thermohaline circulation in a global ocean GCM, as a function of the surface freshwater flux. The central feature is a saddle-node bifurcation first described by Stommel⁵ in 1961; it is the point beyond which the NADW circulation cannot be sustained, wind-

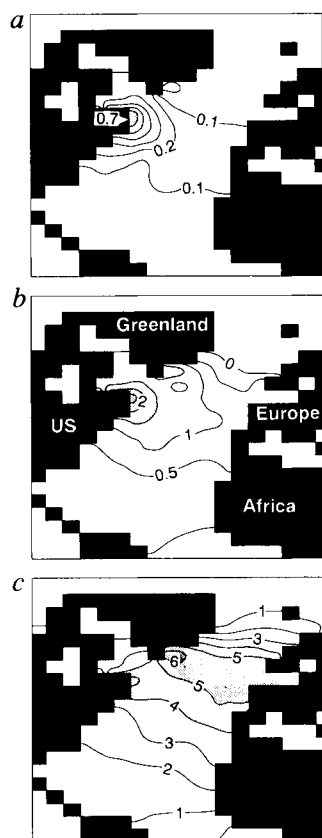


FIG. 6 Sea surface temperature change (in °C) for three types of NADW circulation changes. a, Difference between warm and cold phase of the inter-decadal oscillation; b, difference between 'normal' and 'weak' NADW equilibria (states a minus c of Fig. 2); c, difference between 'normal' and absent NADW circulation (states a minus f of Fig. 2). Shading highlights regions of strongest sea surface temperature change; states with stronger NADW overturning are warmer in each case.

ing down on an advective timescale of centuries. The spin-down is characterized by gradually weakening heat transport and convection, and must be distinguished from the dramatic instability which occurs when convection is suddenly interrupted in the entire North Atlantic—a much faster process known as 'polar halocline catastrophe'. Due to the slow forcing change this kind of collapse, which may be temporary or permanent, was not observed in the experiments reported here, but it has been found in models subjected to rapid and massive meltwater discharge^{9,22,39}.

Stommel's bifurcation is a very robust feature, found in models of different complexity—from simple box models to GCMs. In our GCM, the critical point is reached for an additional freshwater input of only 0.06 Sv into the NADW catchment area. This is much less than a previous estimate of 0.3 Sv, which was obtained with an idealized two-dimensional model without wind forcing¹⁰. The new estimate is an uncomfortably small perturbation to the present hydrological cycle (compare Fig. 1b). It is impossible to give an error margin on this estimate, but it depends not on regional details, only on the basin-scale circulation, which the model reproduces quite well. A slow spin-down, which seems to be of the type discussed here, was found in a coupled model scenario¹⁷ for a quadrupling of atmospheric CO₂.

A second feature of the bifurcation map is the existence of multiple states with different rates of NADW formation. Transitions between these states can be triggered for even smaller, regional changes in the freshwater budget, and they can lead to substantial SST changes within a few years. In this model, the major transition is between states with and without convection in the Labrador Sea. This result, however, depends on regional details of convection, which are not properly resolved in a coarse model; the physical principle is likely to be robust, although the specific details may not be. In the real world, a substantial part of NADW is formed north of Iceland, but present climate models have trouble representing the physics of the overflow over the Greenland–Scotland ridge. There is good evidence that convection north of Iceland shut down in glacial times¹⁵. It is possible that this convection region is more vulnerable to climate change than the one in the Labrador Sea; this cannot be determined using the present model. The freshwater balance of this convection region can be upset by precipitation changes, by melting ice (melting Arctic sea ice at a rate of 5 cm yr⁻¹ would provide a freshwater flux of ~0.01 Sv), but also by changes in regional currents. For example, the East Greenland current presently removes some of the net precipitation falling over the Arctic seas, transporting it southwards over the sill³⁶. It is therefore difficult to establish how vulnerable the present convection pattern is to climate changes, or indeed whether deep convection north of Iceland has ceased already³⁷.

The third feature is the existence of the Hopf bifurcation, leading to an oscillation of the meridional overturning rate centred on the Labrador region. This bifurcation suggests that there are parameter ranges where the ocean circulation is intrinsically steady and would vary only in response to variable atmospheric forcing, and other parameter regions where the ocean generates self-sustained interdecadal oscillations. This opens the possibility that a change in hydrological forcing can shift the North Atlantic from a stable climate to an oscillatory regime, or vice versa. It will be an important topic of future research to establish the exact forcing conditions under which oscillations occur. □

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A homeobox gene essential for zebrafish notochord development

William S. Talbot^{*}, Bill Trevarrow^{†§}, Marnie E. Halpern^{‡§}, Anna E. Melby^{*}, Gist Farr[†], John H. Postlethwait^{*}, Trevor Jowett[†], Charles B. Kimmel^{*} & David Kimelman^{‡||}

^{*} Institute of Neuroscience, University of Oregon, Eugene, Oregon 97403, USA

[†] Department of Biochemistry and Genetics, University of Newcastle upon Tyne, Newcastle upon Tyne NE2 4HH, UK

[‡] Department of Biochemistry, Box 357350, University of Washington, Seattle, Washington 98195-7350, USA

The notochord is a midline mesodermal structure with an essential patterning function in all vertebrate embryos. Zebrafish *floating head* (*flh*) mutants lack a notochord, but develop with prechordal plate and other mesodermal derivatives, indicating that *flh* functions specifically in notochord development. We show that *floating head* is the zebrafish homologue of *Xnot*, a homeobox gene expressed in the amphibian organizer and notochord. We propose that *flh* regulates notochord precursor cell fate.

A CASCADE of inductive signals establishes the vertebrate body plan. The organizer¹, a region of dorsal mesoderm with the remarkable ability to induce the formation of a second embryonic axis when transplanted to the ventral region of a host embryo, provides a dramatic example of one such inductive interaction. Cells in the organizer region at the beginning of gastrulation later form mesodermal structures that occupy the midline of the animal, including the prechordal plate anteriorly and the notochord posteriorly^{2,3}. These organizer derivatives have distinct signalling capacities that appear to be required for the proper patterning of adjacent tissues during embryogenesis. For example, the prechordal plate appears to induce forebrain structures in the anterior neural tube, whereas the notochord induces the floor plate in more posterior regions of the central nervous system^{4,6}. Moreover, in the paraxial mesoderm that forms the somites, the notochord induces sclerotome differentiation⁷ and, in zebrafish, formation of the muscle pioneers⁸.

The functional complexity of the organizer and its derivatives is reflected by the expression patterns of several transcription factors, including *goosecoid*⁹, *HNF-3 β /Pintallavis*^{10,13}, *Lim1* (ref. 14), *Brachyury*^{15,17} and *Xnot*^{18,19}. The mouse gene *HNF-3 β* ^{12,13} and its frog homologue *Pintallavis*^{10,11} are expressed throughout the organizer region, and later in both prechordal plate and notochord. Mice bearing *HNF-3 β* loss-of-function mutations lack a notochord and possibly a prechordal plate, indicating that this gene has an essential role in the development of the axial mesoderm^{20,21}. In contrast, other genes are expressed in more limited regions of the axial mesoderm. For example, *goosecoid* is specifically expressed in the anterior axial mesoderm that contributes to the prechordal plate^{9,22}. Conversely, *Brachyury*^{15,17} and *Xnot*^{18,19} are expressed in the more posterior axial mesoderm that forms the notochord. *Brachyury* and its zebrafish homologue *no tail* (*ntl*) are known by mutational analysis to function in notochord development^{8,15,23,24}. Based on its expression pattern, *Xnot* has been suggested to have a role in organizer activity or notochord development¹⁸, but the function of this gene remains unknown.

In zebrafish, genes required for axial development have been identified as embryonic patterning mutations^{8,25–28}. Here we report the isolation and molecular characterization of a new

embryonic lethal mutation, *floating head* (*flh*), which disrupts axial mesoderm development. The notochord does not form in *flh* mutants, and the somites are fused medially under the neural tube; *flh* mutants also have defects in ventral cell types within the neural tube that are induced by the notochord. We show that *flh* is a homeobox gene homologous to *Xnot* that is expressed in the zebrafish organizer at the beginning of gastrulation, and later in the developing notochord. These results demonstrate that *flh* is essential for the development of the notochord, and we suggest that *flh* regulates cell fate decisions in the organizer.

Lack of a notochord in *flh* mutants

We discovered the original *flh* allele, *flh*^{nl}, as a spontaneous mutation in an outbred strain of fish obtained commercially. We identified a second allele, *flh*^{b327}, in a screen for γ -ray-induced mutations that fail to complement *flh*^{nl}. Both *flh* alleles segregate as mendelian recessive embryonic lethal mutations, and the molecular analysis described below suggests that both are *flh*-null alleles. During gastrulation and segmentation, *flh*^{nl} and *flh*^{b327} homozygotes have similar phenotypes but, in contrast to *flh*^{nl} homozygotes, *flh*^{b327} embryos display extensive necrosis by 24 hours post-fertilization (h). We present evidence that *flh*^{b327} is a deficiency; hence the necrosis in 24-h *flh*^{b327} homozygotes probably results from the deletion of one or more essential genes closely linked to *flh*.

We show that *flh* mutants lack a differentiated notochord (Fig. 1), indicating that *flh*⁺ function is essential for notochord development. In wild-type embryos, the notochord contains large cells with a characteristic vacuolated morphology (Fig. 1c). These cells are absent in *flh* mutants (Fig. 1d). In addition, we examined the expression of several notochord markers in *flh* mutants (Figs 1 and 2). An antibody against keratan sulphate labels the circumference of the notochord in wild-type embryos^{29,30} (Fig. 1c), but no notochord staining is evident in *flh* mutants (Fig. 1d). In addition to the notochord, *flh*^{nl} embryos lack a hypochord and the blood vessels that normally develop ventral to the notochord, the dorsal aorta and cardinal vein (Fig. 1d and data not shown). In the trunk of *flh* mutants, left and right somites, which are paired bilaterally in wild-type embryos (Fig. 1c, e), fuse under the spinal cord to form single, unpaired myotomes (Fig. 1d, f). Fusion of the trunk somites occurs at or soon after segmentation (Fig. 1f).

In contrast to the notochord, development of the prechordal plate is not disrupted in *flh* mutants (Fig. 1). The presence of a prechordal plate in *flh* mutants is indicated both by *goosecoid*²²

§ Present addresses: Division of Biology 139-74, Caltech, Pasadena, California, 91125, USA (B.T.); Carnegie Institution of Washington, Department of Embryology, 115 West University Parkway, Baltimore, Maryland 21210, USA (M.E.H.).

|| To whom correspondence should be addressed.

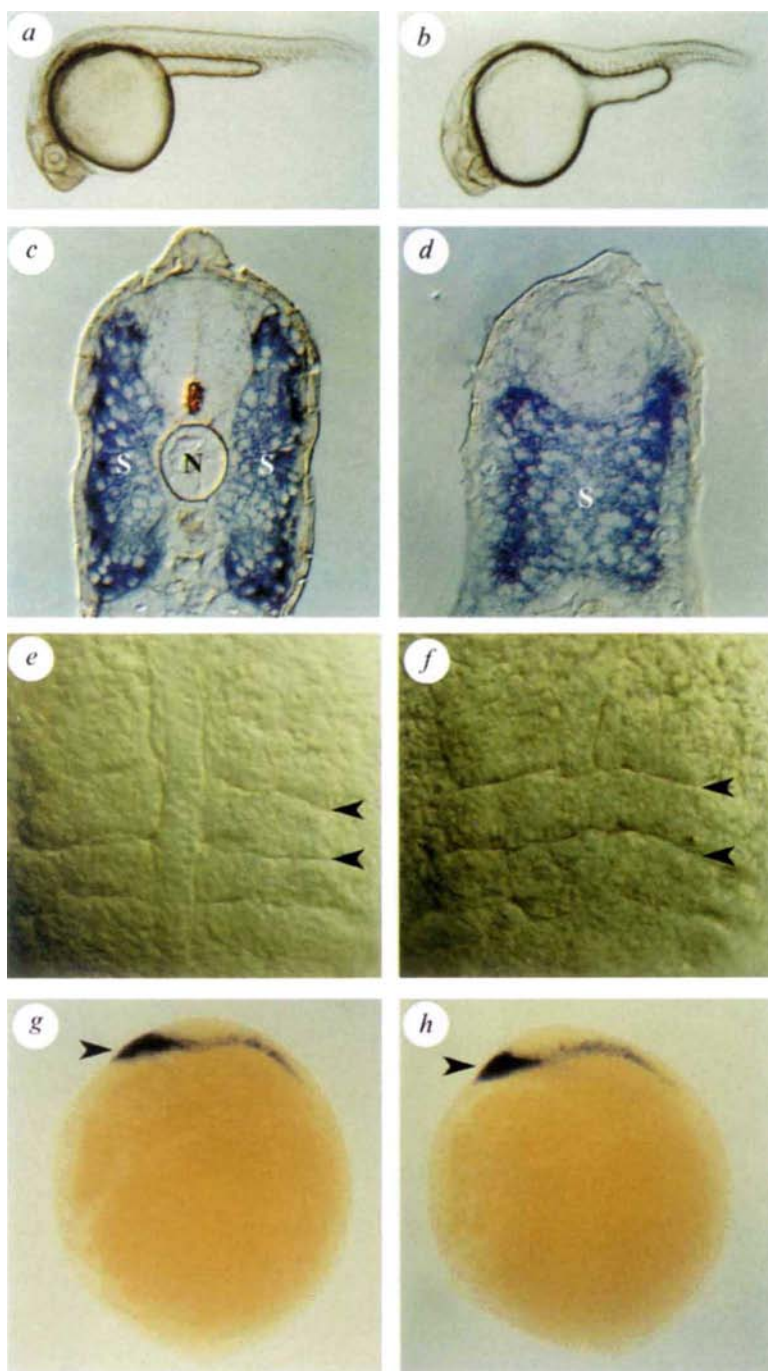


FIG. 1 *flh* mutants lack a notochord. Comparison of **a**, wild-type, and **b**, *flh*^{nt} embryos at 24 hours of development (h). In addition to the notochord, *flh*^{nt} embryos lack a hypochord and the blood vessels that normally develop ventral to the notochord, the dorsal aorta and cardinal vein (data not shown). Although the body axis is abnormally short, *flh*^{nt} animals have the normal number of somites. **c**, **d**, Expression of keratan sulphate (brown stain) and the muscle-specific α -tropomyosin mRNA (blue stain) in double-label preparations of 24-h wild-type (**c**) and *flh*^{nt} (**d**) zebrafish embryos. Transverse sections from the trunk are shown. In wild-type embryos (**c**), the anti-keratan sulphate antibody MZ15 (ref. 30) labels the circumference of the notochord (N), in addition to the apical surface of the floor plate and the central canal in the CNS. No notochord staining is evident in *flh* mutants (**d**). Similar results were obtained with other notochord markers, including α -collagen II (refs 44, 45) and the znt-1 antigen (B.T., unpublished results). The α -tropomyosin⁴⁶ (B. Riggleman, personal communication) staining reveals that bilaterally paired somites (S) are separated by the notochord in wild-type embryos (**c**), whereas a single fused somite is present in the position of the notochord in *flh*^{nt} mutants (**d**). In contrast to the trunk, tail somite fusion is variable in *flh*^{nt} mutants: tail somites usually form unfused pairs, resulting in bilaterally separate myotomes (not shown). As in the trunk, there is no notochord in the *flh*^{nt} tail, and the space between the unpaired tail somites is often filled by an accumulation of blood. Notochords are not present in *flh*^{b327} homozygotes, which typically have fused somites in both the trunk and tail (data not shown). **e**, **f**, Dorsal views (anterior to the top) of wild-type (**e**) and *flh*^{nt} mutant (**f**) embryos at the 3-somite stage (11 h). Arrows mark the borders of the second somites, which are separated by the notochord in the wild-type and fused across the midline in the mutant. In *flh* mutants, some recently formed trunk somites are separate (see, for example, the first somite pair in **f**); these typically fuse by the end of the segmentation period (24 h). **g**, **h**, Whole-mount analysis of *goosecoid* (*gsc*) expression in wild-type (**g**) and *flh* mutant (**h**) embryos at the bud stage (10 h). *flh* mutants have a prominently labelled polster (arrow).

METHODS. Embryos were staged as described⁴⁷. For **c** and **d**, whole-mount *in situ* hybridization⁴⁶ with the α -tropomyosin probe was followed by immunohistochemical staining⁸ with MZ15 (ref. 30). The stained embryos were embedded in Epon and sectioned. For staining with the *gsc* probe²², wild-type and *flh*^{nt} siblings were identified by the presence or absence of the notochord rudiment before fixation.

expression indistinguishable from wild-type, and by the formation of a polster (pillow), a prominent mass of prechordal plate cells under the most anterior neural plate (Fig. 1*h*). Moreover, *flh* mutants have hatching glands (not shown), which derive from prechordal axial cells in the polster³. In addition, derivatives of the nonaxial mesoderm, such as muscle, heart, pronephros and blood, are present in *flh* mutants (Fig. 1 and data not shown). Thus *flh*⁺ function is not generally required for the development of mesodermal derivatives; rather, the mutant phenotype suggests that *flh*⁺ functions specifically in notochord development.

Floor plate and motor neurons

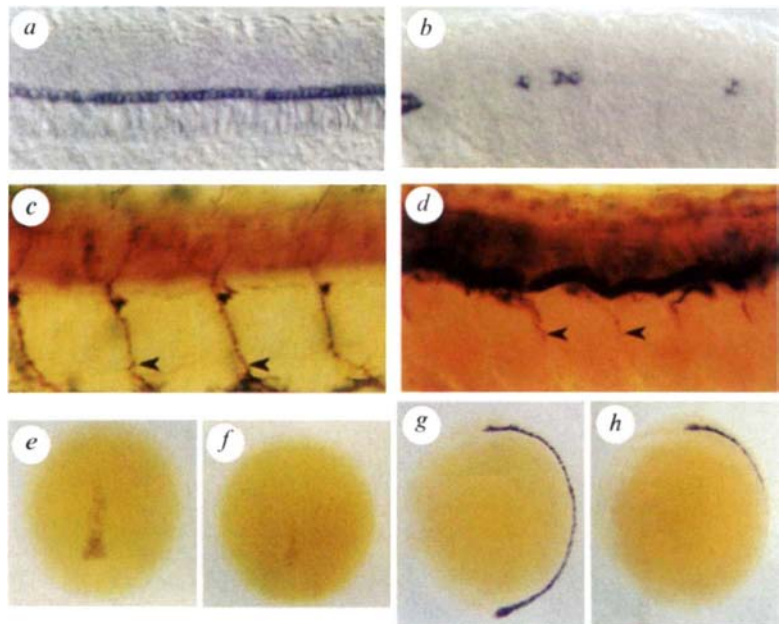
In the ventral neural tube, the notochord induces the differentiation of the floor plate and adjacent motor neurons^{5,6,31}. We therefore examined these cell types in *flh* mutants. Despite the absence of a differentiated notochord, floor-plate cells are present in a continuous row at the ventral midline of the midbrain, hindbrain

and anterior spinal cord (Fig. 2*h* and data not shown). In the spinal cord of the posterior trunk and tail, the floor plate is disrupted, with floor-plate cells being variably located in discontinuous groups (Fig. 2*b*).

Some primary and secondary motor neurons are also present in *flh* mutants (Fig. 2*d*). The axons of these neurons are abnormal (Fig. 2*d*), perhaps reflecting the inability of these axons to navigate the fused, disorganized muscle masses present in these animals.

The finding that some floor-plate cells and motor neurons are present in *flh* mutants might indicate that axial mesodermal cells in these animals can induce floor-plate and motor neuron differentiation for a limited time during gastrulation, even though these mesodermal cells do not differentiate as notochord later in embryogenesis. Consistent with this possibility, dorsal marginal cells in *flh* mutant gastrulae transiently express *sonic hedgehog* (*shh*) (Fig. 2*f*), which encodes a secreted protein that can

FIG. 2 Floor plate and motor neurons are disrupted in *flh* mutants. *a, b*, *sonic hedgehog* (*shh*)³⁴ expression in the floor plate of a wild-type embryo (*a*) and in discontinuous clusters of floor plate cells in a *flh*ⁿ¹ mutant (*b*): both panels show staining in the spinal cord of 22 h embryos oriented with the anterior to the left and the dorsal side up. Similar results were obtained with two additional floor-plate markers, α -collagen II (refs 44, 45) and the zn-5 antigen⁴⁸ (B.T., unpublished observations). At this stage, *shh* is also expressed in the posterior tail notochord of wild-type embryos³⁴, but the length of notochord shown in *a* is from the trunk and is therefore not labelled. *c, d*, Labelling of the ventral nerves (arrows) with the znp-1 monoclonal antibody⁴⁸ in wild-type (*c*) and *flh*ⁿ¹ mutant (*d*) embryos. Both panels show 36-h embryos oriented as above. In contrast to the wild-type, the mutant has stunted, irregular, ventral nerve axons. At this stage, wild-type ventral nerves contain axons from both primary and secondary motor neurons^{49,50}. In *flh* mutants, some primary and secondary neurons are present, as demonstrated by zn-1 (refs 48, 49) and zn-5 (refs 48, 50) staining, respectively (not shown), and axons from both cell types apparently contribute to the abnormal ventral nerves seen in these animals (*d*). *e-h*, *shh* expression in whole-mount preparations. *e, f*, Dorsal views (animal pole to the top) of wild-type (*e*) and *flh*ⁿ¹ (*f*) embryos at midgastrulation (80% epiboly, 8.3 h). *g, h*, Side views (anterior to the top) of wild-type (*g*) and *flh*ⁿ¹ (*h*) embryos at bud stage (10 h).



METHODS. Whole-mount *in situ* hybridization with the *shh* probe³⁴ was performed as described⁴⁶. Immunohistochemistry was performed as described⁴⁸.

induce floor plate and motor neurons^{32,34}. In wild-type embryos at midgastrulation, *shh* is expressed in mesodermal cells occupying approximately the posterior half of the developing embryonic axis³⁴ (Fig. 2*e*); *shh* is expressed in *flh* mutants at this stage, but in a more limited domain located near the blastoderm margin (Fig. 2*f*). In wild-type embryos at the end of gastrulation (10 h), *shh* is expressed in posterior, but not anterior, axial mesoderm and in the ventral neural plate all along the anteroposterior axis³⁴ (Fig. 2*g*). At this stage, *shh* messenger RNA is no longer detectable in the axial mesoderm of *flh* mutants (Fig. 2*h*). There appears to be no disruption of *shh* expression in the anterior neural plate, but expression in the posterior neural plate is patchy (Fig. 2*h*), presumably reflecting the floor-plate cell clusters apparent in older embryos.

A zebrafish *Xnot* homologue maps near *flh*

Because *flh*⁺ function is essential for notochord development, candidate genes for *flh* include those expressed in the developing notochord. Because *Xnot* is expressed in the notochord precursors in *Xenopus*¹⁸, we examined the possibility that *flh* is the zebrafish homologue of *Xnot*. To isolate zebrafish genes related to *Xnot*, we used polymerase chain reaction (PCR) with degenerate oligonucleotide primers designed to hybridize preferentially to the homeoboxes of *Xnot* and the related *Drosophila* gene *90Bre*³⁵. Amplification of RNA from mid-gastrula zebrafish embryos yielded a variety of products, one of which represented an *Xnot* homologue. We show that this gene is *flh*.

We isolated several complementary DNA clones corresponding to the PCR amplification product from gastrula- and segmentation-stage libraries³⁶. Sequence analysis of the longest cDNA clone revealed an open reading frame (ORF) of 241 amino acids that contains a homeobox sequence (Fig. 3*a*). The homeodomain sequence places the gene in the *ems* subfamily (Fig. 3*b*), which includes *Xnot*^{18,19}, the chicken gene *Cnot*³⁷, the *Drosophila* genes *empty spiracles* (*ems*)³⁸ and *90Bre*³⁵, and the *Emx-1* and *Emx-2* genes of mouse and human³⁹. Of these, the zebrafish homeodomain sequence is most closely related to those encoded by *Xnot* (75% identity) and *Cnot* (72% identity), suggesting that these genes are orthologous.

As a test of the hypothesis that the zebrafish *Xnot* homologue is *flh*, we determined whether the cloned gene is tightly linked to the mutation in the genetic map⁴⁰. As the first step in this analysis, we mapped *flh* by identifying nearby random amplified polymorphic DNA (RAPD) markers, which are mendelian DNA polymorphisms amplified by single decamer primers of arbitrary sequence⁴¹. RAPD primers amplify different fragments from different zebrafish strains, because of strain-specific sequence differences in primer binding sites or strain-specific differences in distance between primer binding sites, and hence they reveal polymorphisms between strains.

Using the strategy described previously⁴⁰, we identified a RAPD marker, 7A.1450, on linkage group 13 that is tightly linked to *flh* (Fig. 3*c, d*). In experiments such as that illustrated in Fig. 3*c*, we observed no recombinants among 1,332 haploid embryos in *flh*ⁿ¹ mapping families genotyped for this RAPD marker or for a sequence-tagged site marker derived from it (Fig. 3).

As the second step in the analysis, we searched for a polymorphism linked to the *Xnot* homologue, so that the gene's map position could be identified. We determined the gene's structure and found a polymorphism in one of the two introns: PCR primers that amplify a fragment containing most of the larger intron revealed that intron-length variants of 850 and 830 base pairs (bp) segregate as alleles in the family of haploid embryos in the linkage map cross⁴⁰ (Fig. 3*e*, top).

The embryos in the linkage-map cross were also genotyped for the 7A.1450 RAPD marker linked to *flh* (Fig. 3*e*, bottom). The intron-length polymorphism in the *Xnot* homologue co-segregated with the 7A.1450 marker: all individuals displaying the 7A.1450 fragment have the 850-bp intron allele (Fig. 3*e*, +) and individuals lacking the 7A.1450 fragment have the 830-bp intron allele (Fig. 3*e*, -). No recombinants between 7A.1450 and the zebrafish *Xnot* homologue were observed among 87 haploid embryos in the mapping panel (0 ± 2.2 cM). Thus the zebrafish *Xnot* homologue and *flh* both map to the same small region of linkage group 13 identified by the 7A.1450 marker.

Molecular basis of *flh* mutations

Additional evidence that the zebrafish *Xnot* homologue is *flh* derives from an analysis of molecular lesions involving the *Xnot*

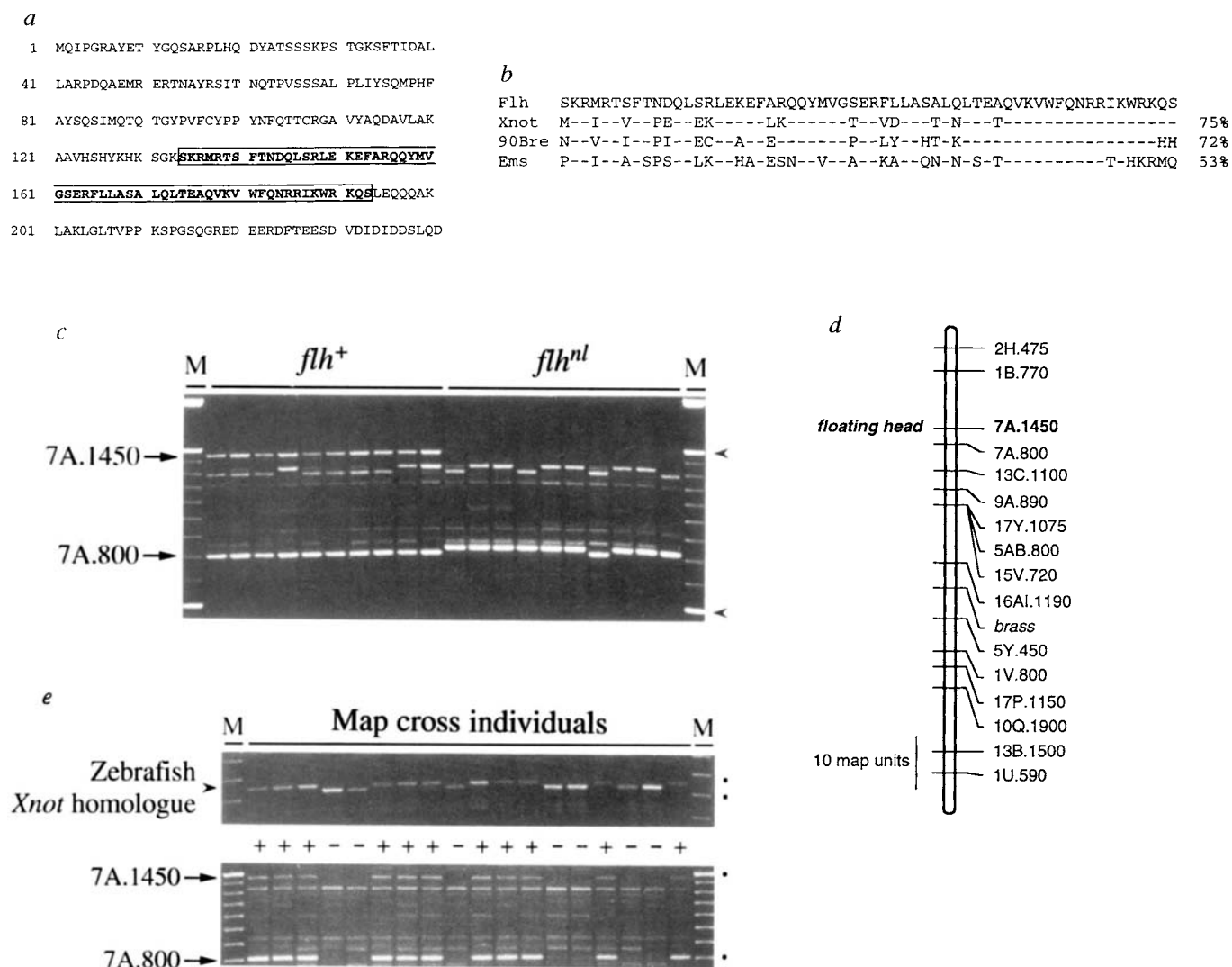


FIG. 3 A zebrafish homeobox gene homologous to *Xnot* maps at or near *flh*. **a**, Predicted amino acid sequence of the *Xnot* homologue. The sequence was submitted to GenBank (accession number L48017). We show below that this gene is *flh*. The homeodomain is boxed. The open reading frame (ORF) is likely to be complete, as there is a stop codon 12 bp upstream of the putative start codon. **b**, Sequence alignment of homeodomains of Flh and other members of the Ems homeodomain subfamily. Dashes indicate residues identical to the Flh homeodomain. There is little conservation between Flh and other Ems subfamily proteins outside the homeodomain. **c**, Identification of a RAPD marker that is tightly linked to *flh*. Ten *flh*⁺ and ten *flh*^{-/-} haploid embryos were genotyped with the A7 RAPD primer, which amplifies two markers linked to *flh*, 7A.1450 and 7A.800, as well as several unlinked markers. The embryos were obtained from a single female that was heterozygous for *flh*^{-/-} and for the two linked RAPD markers. Thus the *flh*⁺ and *flh*^{-/-} alleles and the RAPD alleles are present in half of the haploid embryos in the mapping family. 7A.1450 is linked to *flh*, as all of the embryos that inherited the *flh*⁺ allele also inherited the 7A.1450⁺ allele (that is, the 7A.1450 fragment is amplified from the genomic DNA of all of the wild-type embryos), and all of the *flh*^{-/-} individuals are 7A.1450⁻. In similar experiments, we observed no recombinants among 1,332 haploid embryos in *flh*^{-/-} mapping families genotyped for the 7A.1450 RAPD marker or a sequence-tagged site marker derived from it, indicating that *flh* and 7A.1450 are separated by only a very small genetic distance (0 ± 0.15 cM). 7A.800 is also linked to *flh*, as the ten *flh*⁺ animals have the 800-bp allele, and nine of ten mutants have the 810-bp allele. The *flh*^{-/-} animal with the 800-bp allele of 7A.800 is a recombinant (seventh mutant lane). Arrowheads indicate size standards of 1,500 bp and 600 bp in the marker lanes (M). **d**, Genetic map of linkage group 13 (LG 13; from ref. 40 and this work), showing the positions of *flh*, 7A.1450 and 7A.800. **e**, The zebrafish *Xnot* homologue co-localizes with 7A.1450 and, therefore, with *flh*. Individual haploid embryos in the linkage map family⁴⁰ were genotyped for an intron-length polymorphism in the *Xnot* homologue (top) by scoring alleles of 830 bp and 850 bp. The same animals were genotyped for 7A.1450 and 7A.800 (bottom). The *Xnot* homologue alleles co-segregate with the 7A.1450 alleles: all of the 7A.1450⁺ individuals (+) have the 850-bp intron-length allele and all of the

7A.1450⁻ individuals (-) have the 830-bp allele. In this cross, the 7A.800 marker is scored by the presence or absence of the 800-bp fragment, rather than by a size difference, as in the *flh* mapping cross above. This results from differences in the genetic backgrounds of the strains used in the two crosses. Dots in the upper panel indicate size standards of 900 bp and 800 bp in the marker lanes, and, in the lower panel, size standards of 1,500 bp and 800 bp. **METHODS.** The primers used to amplify the *flh* homeobox fragment were 5'-CA(A/G)CA(A/G)TA(C/T)ATGGTXGG-3' and 5'-CGCGGATCC(T/G)XC(T/G)(A/G)TT(T/C)-TG(A/G)AACCA-3'. PCR with these degenerate primers was conducted in two phases: two cycles with an annealing temperature of 42 °C were followed by 40 cycles with an annealing temperature of 65 °C. The melting and extension temperatures were 94 °C and 72 °C, respectively, in both phases of the PCR. The PCR products were cloned and sequenced, and one that represented a possible zebrafish *Xnot* homologue was used as a probe to identify cDNA clones from gastrula- and segmentation-stage cDNA libraries³⁶. The sequence of the Flh protein shown in **a** derives from the longest of these cDNA clones. The 7A.1450 RAPD marker linked to *flh* was identified by screening genomic DNA samples in two pools, one from *flh*⁺ animals, the other from *flh*^{-/-} siblings, in PCR with decamer primers of arbitrary sequence. The use of this method, termed bulked segregant analysis⁵¹, to map zebrafish mutations has been described previously⁴⁰. The sequence of the A7 RAPD primer (Operon Technologies) is 5'-GAAACGGGTG-3'; RAPD markers were named as described previously⁴⁰. Some individuals in *flh*^{-/-} mapping families were genotyped with a sequence-tagged site (STS) marker derived from the 7A.1450 RAPD marker. To generate this STS marker, the 7A.1450 RAPD fragment was cloned and sequenced, and primers (5'-GAAACGGGTGTATATAATAA-3' and 5'-GATTTTGTACAGAGATTATAA-3') that specifically amplify this fragment were developed. These primers amplify a fragment of similar size from both wild-type and *flh*^{-/-} animals in the mapping families, but a polymorphism is evident when the amplification products are cleaved with the restriction endonuclease *MspI*. To score the intron-length polymorphism in the zebrafish *Xnot* homologue, the primers 5'-GTAATG-GCGAAAGCAGCAGTT-3' and 5'-GTTATG-AAACGCTTTGCAG-3' were used to amplify a fragment containing most of the second intron from the individual DNA samples in the linkage map panel⁴⁰.

homologue in four independent *flh* mutations. We found that *flh*^{b327}, a γ -ray-induced mutation, is a deficiency that deletes the candidate gene: three different pairs of PCR primers corresponding to the zebrafish *Xnot* homologue amplified the expected fragment from the genomic DNA of *flh*⁺ haploid embryos, but not from their *flh*^{b327} siblings (Fig. 4a, left, and data not shown). Control primers for *shh*³⁴ amplified the expected product from both *flh*⁺ and *flh*^{b327} DNAs (Fig. 4a, right). In Southern blot assays, a probe synthesized from the *Xnot* homologue cDNA did not hybridize to any fragments of *Eco*RI-cleaved *flh*^{b327} genomic DNA, confirming that the gene is deleted (data not shown).

We analysed three other *flh* mutations and found that they contain small lesions within the candidate gene. Fragments containing coding sequence from the zebrafish *Xnot* homologue were amplified from genomic DNA isolated from *flh*ⁿ¹ haploid embryos and their *flh*⁺ siblings, and the PCR products were cloned and sequenced. A deletion of two base pairs is evident in the sequence derived from *flh*ⁿ¹ DNA (Fig. 4b). This deletion causes a frameshift in the ORF and leads to truncation of the *flh*ⁿ¹ polypeptide upstream of the homeodomain (Fig. 4c). We also analysed two ENU-induced *flh* alleles, *flh*^{tk241} and *flh*^{tm229}, that were isolated in a screen for embryonic patterning

mutations²⁷ (P. Haffter and C. Nüsslein-Volhard, personal communication). Each has a different base-substitution mutation that introduces a premature translation termination codon in the ORF (Fig. 4c).

We conclude that the candidate gene we identified by homology to *Xnot* is *floating head*, based on its genetic co-localization with the *flh*ⁿ¹ mutation (Fig. 3) and the characterization of the molecular lesions in four *flh* alleles (Fig. 4). Because none of the four mutant alleles is predicted to encode a full-length, homeo-domain-containing protein, it is likely that they are all *flh*-null mutations.

Expression of *flh* mRNA

The absence of notochord in *flh* mutants suggests that *flh*, like its frog and chick homologues^{18,19,37}, is expressed in notochord precursors. We examined the expression pattern of *flh* mRNA during early embryogenesis by *in situ* hybridization with a *flh* cDNA probe. *flh* mRNA is first expressed at detectable levels in the marginal cells of the late blastula (4.3 h) (Fig. 5a, b). At this stage, *flh* expression is evident in cells around the entire circumference of the margin, but more *flh*-expressing cells are located on the dorsal side of the embryo than elsewhere. This pattern is refined until the early gastrula stage (6 h), when the

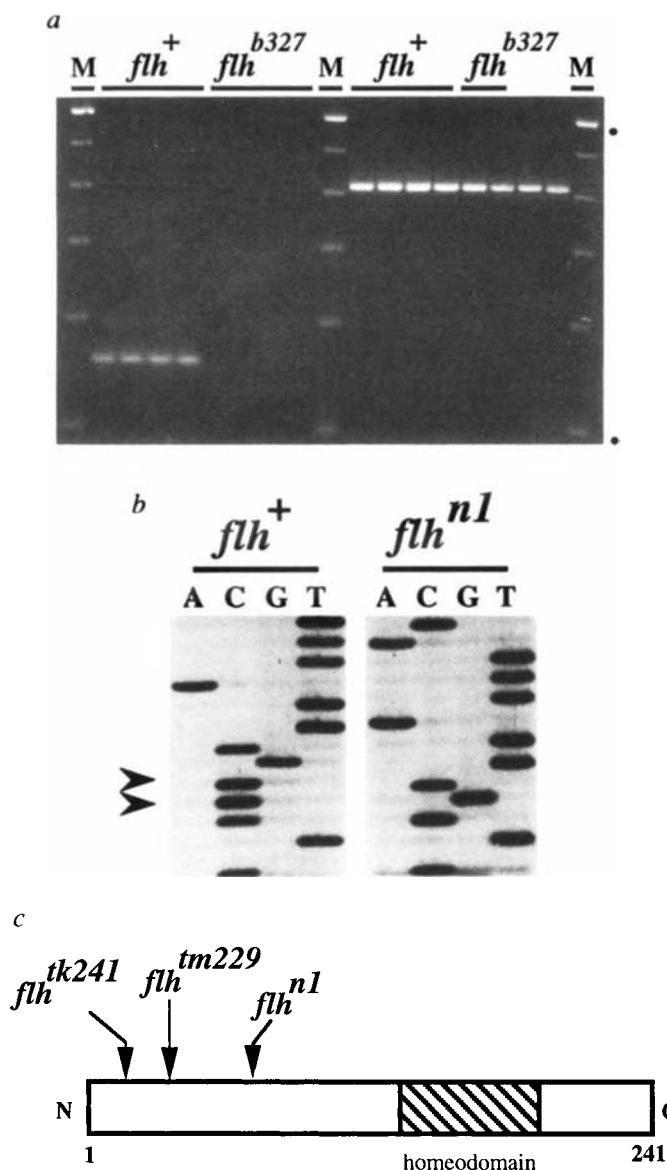


FIG. 4 Identification of molecular lesions in *flh* mutations. **a**, The zebrafish *Xnot* homologue is deleted in *flh*^{b327}. Primers corresponding to zebrafish *Xnot* homologue (left) amplify the expected fragment from the genomic DNA of four *flh*⁺ individuals, but not from four *flh*^{b327} siblings. This result was obtained with three different pairs of primers corresponding to the candidate gene. The same genomic DNA samples were used in PCR with *sonic hedgehog* primers (right), and the expected fragment is amplified from *flh*⁺ and *flh*^{b327} preparations. Dots mark size standards of 600 bp and 100 bp in the marker lanes (M). **b**, The *flh*ⁿ¹ mutation is a 2-bp deletion in the zebrafish *Xnot* homologue ORF. Fragments containing coding sequence were amplified from both *flh*⁺ and *flh*ⁿ¹ genomic DNA. The PCR products were cloned and sequenced, and a portion of a sequencing gel is shown. The two nucleotides marked by arrowheads in the *flh*⁺ sequence are absent in the *flh*ⁿ¹ sequence. **c**, A schematic representation showing the predicted Flh protein and the positions of mutations in *flh*ⁿ¹ and two ENU-induced alleles, *flh*^{tm229} and *flh*^{tk241}. All three alleles truncate the Flh protein upstream of the homeodomain. In *flh*ⁿ¹, two C residues at positions 211 and 212 of the wild-type coding sequence are deleted; this results in a frameshift after the leucine residue at position 70 in the ORF. The *flh*^{tm229} mutation has a T in place of an A at position 97 of the coding sequence; this changes codon 33 of the wild-type ORF (AAA; Lys) to a stop codon (TAA). The *flh*^{tk241} mutation has an A in place of a T at position 53 of the coding sequence; this changes codon 18 of the wild-type ORF (TTG; Leu) to a stop codon (TAG). Fragments representing the entire *flh*ⁿ¹ ORF were sequenced, and, other than the mutation, there were no differences between it and the wild-type cDNA sequence. Fragments representing the first 177 codons of the *flh*^{tk241} and *flh*^{tm229} ORFs were sequenced; in addition to their respective mutations, these two alleles have a polymorphism (codon 64 is TCT, Ser) relative to the sequences of the cDNA and *flh*ⁿ¹ (codon 64 is CCT, Pro).

METHODS. The primers in **a** derive from the coding strand of the first exon (5'-CGTGGAGCTGTGTACGCA-3') and the non-coding strand of the second exon (5'-AACTGCTGCTTTCGCCAG-3'). The length of the expected amplification product is 156 bp. The sequences of the *shh*³⁴ primers are 5'-TACTGGCGTCTGTACGC-3' and 5'-GGCTCCATTATCTTTGAAAA-3'; the length of the amplified fragment is 413 bp. The mutations in *flh*ⁿ¹, *flh*^{tm229} and *flh*^{tk241} were identified by amplifying fragments containing *flh* coding sequence from genomic DNA by PCR and sequencing the cloned amplification products. For each of the three mutant alleles, at least two clones derived from independent amplification reactions were sequenced to demonstrate that the mutations were not PCR artefacts. The *flh*ⁿ¹ sequences were amplified from DNA samples obtained from haploid animals, and the *flh*^{tm229} and *flh*^{tk241} sequences were amplified from DNA isolated from homozygous mutant diploid animals (genomic DNA samples from *flh*^{tm229} and *flh*^{tk241} were kindly provided by P. Haffter and C. Nüsslein-Volhard).

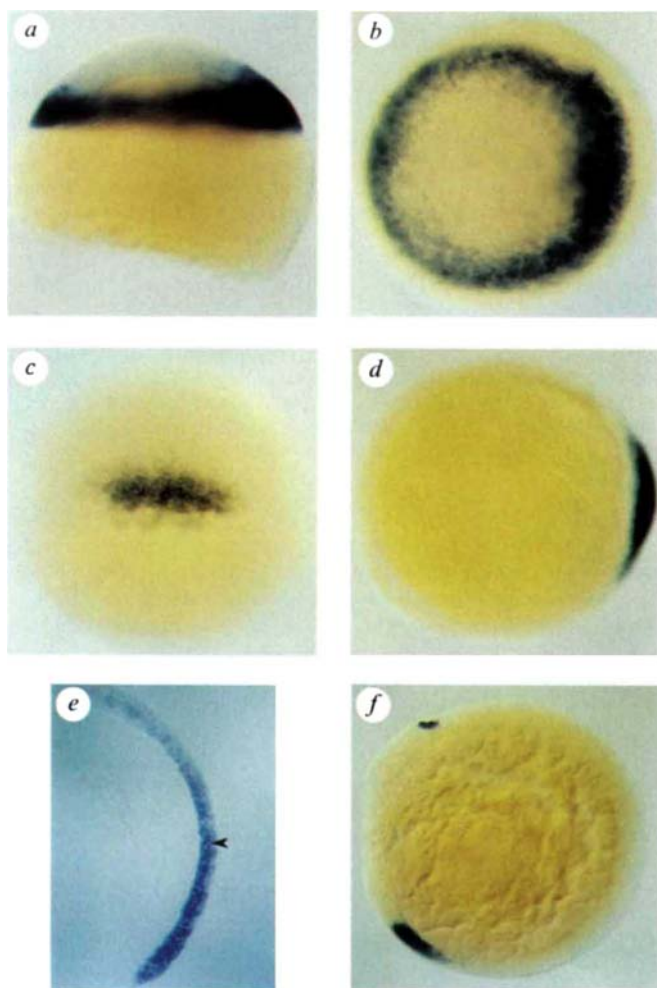


FIG. 5 *flh* mRNA is expressed in the organizer and notochord. Cells expressing *flh* mRNA were visualized by RNA *in situ* hybridization. *a*, *b*, Side view (*a*; animal pole to the top) and animal pole view (*b*; dorsal to the right) at dome stage (4.3 h). *c*, *d*, Dorsal view (*c*; animal pole to the top) and animal pole view (*d*; dorsal to the right) at the onset of gastrulation (shield stage, 6 h). Cells expressing *flh* are localized in the embryonic shield, a thickened marginal region spanning the dorsal midline (visible at the right in *d*). The notochord precursors are located within this dorsal marginal domain of *flh* expression³ (A.E.M., R. M. Warga and C.B.K., manuscript submitted). *e*, Sagittal section at the end of gastrulation (bud stage, 10 h), dorsal to the right, animal pole to the top. Cells expressing *flh* are in both the hypoblast (presumptive mesendoderm) and the epiblast (presumptive ectoderm). The arrow marks the border between the epiblast and hypoblast. Hypoblast and epiblast cells near the developing tail bud express *flh* at similar levels, but, more anteriorly, hypoblast cells express higher levels of *flh* mRNA than epiblast cells. *f*, Side view at the 9–10 somite stage (14 h). *flh* is expressed in the most posterior notochord and overlying neural keel. Anteriorly, *flh* is expressed in what appears to be the rudiment of the dorsal diencephalon.

METHODS. Whole-mount RNA *in situ* hybridization was performed as described⁴⁶. An antisense digoxigenin-labelled riboprobe was synthesized from a clone containing a *flh* cDNA in the pBluescript SK(+) vector (Stratagene). The clone was linearized with *EcoRI* and the probe was synthesized with T7 RNA polymerase. For *e*, a stained whole-mount preparation was embedded in Epon and sectioned.

dorsal marginal cells in the embryonic shield are the only ones expressing *flh* mRNA at a detectable level (Fig. 5*c, d*). The embryonic shield in teleosts is comparable to the amphibian organizer^{42,43}, and the notochord precursors are located within it³ (A.E.M., R. M. Warga and C.B.K., manuscript in preparation).

In the developing trunk axis at the end of gastrulation, *flh* mRNA is expressed weakly in the midline of the neural plate and more strongly in the underlying notochord rudiment (Fig. 5*e*). As somitogenesis begins (10–11 h), *flh* mRNA levels decrease in the anterior notochord, so that only the most posterior notochord cells express *flh* mRNA by midsegmentation (Fig. 5*f*). This expression pattern is very similar to that of *Xnot* in the frog^{18,19} and *Cnot* in the chick³⁷, except that *Xnot* is initially expressed at a high level throughout the blastula¹⁸. Two regions of *flh* expression are apparent in the head primordium during somitogenesis (Fig. 5*f* and data not shown): *flh* is expressed transiently in the polster and throughout somitogenesis in a region of the dorsal diencephalon that includes the epiphysis. It has recently been shown that neurogenesis in this latter region is disturbed in *flh* mutants (S. Wilson, personal communication).

Interaction between *flh* and *no tail*

The finding that *flh* encodes a putative transcription factor essential for notochord development prompted an examination of its interaction with *no tail* (*ntl*), which also functions in the developing notochord^{8,23}. The *ntl* gene is the zebrafish homologue of the mouse gene *Brachyury* (*T*)¹⁵, and, like their murine counterparts²⁴, *ntl* mutants lack a differentiated notochord⁸. Thus both *flh* and *ntl* mutations disrupt notochord development, but the *flh* and *ntl* phenotypes are distinct: in the trunk of *ntl* mutants, mesenchymal cells proposed to be improperly or incompletely differentiated notochord occupy the midline, whereas *flh* mutants have muscle at this location (Fig. 1). Another important phenotypic difference is that *ntl* mutants lack a tail, whereas *flh* mutants do not.

In wild-type embryos, *ntl* is first expressed in cells that will form the germ ring, and later expression is downregulated in cells entering the mesoderm at positions other than the dorsal midline¹⁷ (Fig. 6*a, c, e*). Expression of *ntl* is specifically maintained in the axial mesoderm as cells enter the hypoblast and migrate away from the blastoderm margin, towards the animal pole. By the end of gastrulation, *ntl* expression is confined to the developing notochord cells in the axial mesoderm and to both axial and non-axial cells in the developing tail bud¹⁷ (Fig. 6*e*). In *flh* mutants, *ntl* expression in the germ ring and tail bud does not appear to be altered (Fig. 6*b, d, f*). Expression in the notochord precursors, however, is disrupted. At the end of gastrulation, *ntl* expression is largely absent from the midline of *flh* mutants, so that *ntl* is expressed strongly in the tail bud and faintly in a small axial region immediately adjacent to it (Fig. 6*f*). Earlier in gastrulation, cells expressing *ntl* are scattered along the axis (Fig. 6*b, d*), indicating that at least some axial mesodermal cells in *flh* mutants express *ntl* after leaving the margin to migrate towards the animal pole. These cells apparently do not maintain *ntl* expression, however, as they are not detected at the end of gastrulation (Fig. 6*f*).

These results show that *ntl* expression depends, directly or indirectly, on *flh* function during gastrulation. To learn if the converse is also true, we examined *flh* expression in *ntl* mutants. At the beginning of gastrulation, *flh* expression is not detectably altered in *ntl* mutants (data not shown). At midgastrulation, axial cells in *ntl* mutants express lower levels of *flh* mRNA than those in wild-type embryos (Fig. 6*g, h*). Expression of *flh* mRNA continues at lower, but detectable, levels in *ntl* mutants than in wild-type embryos for the remainder of gastrulation (data not shown). Thus the regulatory interaction between *flh* and *ntl* is complex, and each is required for the maintenance of expression of the other in the developing notochord during gastrulation.

Discussion

We have shown that *flh* encodes a homeodomain protein homologous to *Xnot* in *Xenopus*^{18,19} and *Cnot* in the chick³⁷. Phenotypic analysis of *flh* mutants indicates that *flh* has an essen-

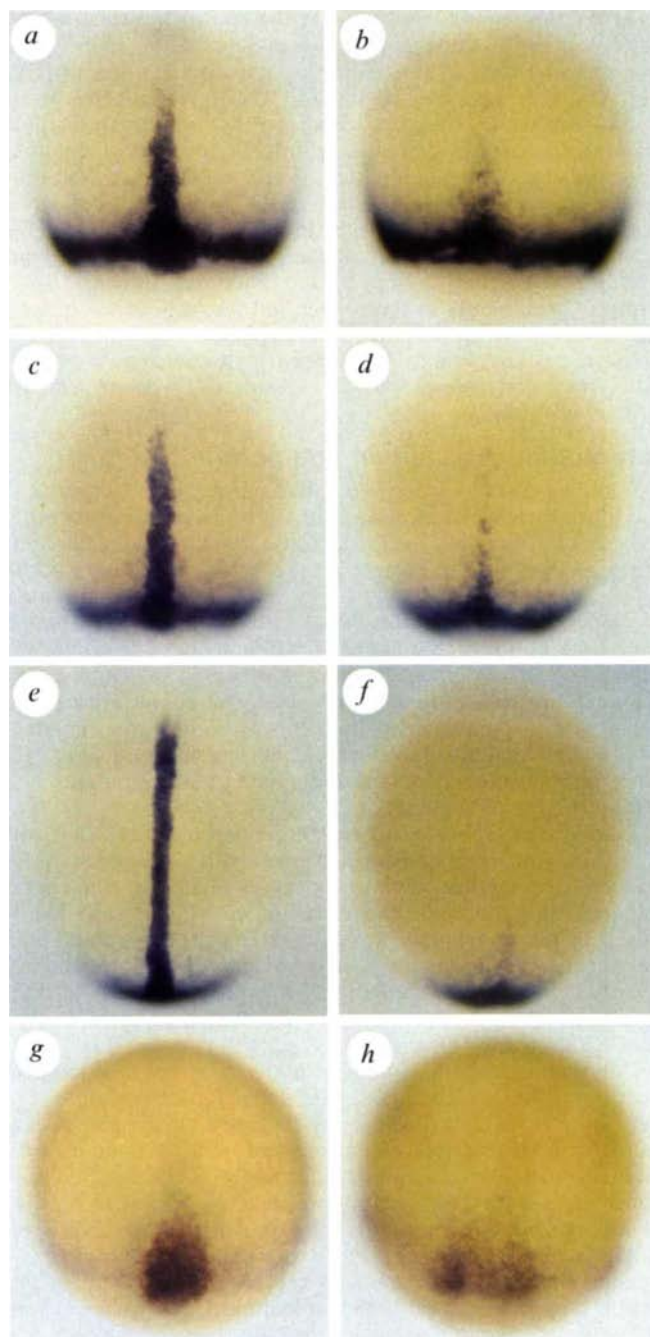


FIG. 6 Interaction between *flh* and *ntl*. Expression of *ntl* (a–f) and *flh* (g, h) mRNA was detected by whole-mount *in situ* hybridization. All are dorsal views, with the animal pole to the top. a–f, *ntl* expression in wild-type (a, c, e) and *flh*^{nt1} (b, d, f) embryos at 80% epiboly (a, b; 8.3 h), 90% epiboly (c, d; 9 h) and bud (e, f; 10 h) stages. In wild-type embryos during gastrulation (a, c), *ntl* is expressed in the germ ring and in the axial mesoderm. In *flh*^{nt1} mutants (b, d), there are fewer labelled cells in the axial mesoderm. In wild-type bud-stage embryos (e), the newly formed tail bud and the developing notochord express high levels of *ntl* mRNA. In *flh*^{nt1} mutants at bud stage (f), the tail bud is strongly labelled, but only a few axial cells near the tail bud are labelled. g, h, *flh* mRNA expression in wild-type (g) and *ntl*^{b195} (h) embryos at 80% epiboly (8.3 h). In *ntl* mutants at midgastrulation (h), *flh* mRNA is expressed more weakly than in wild-type embryos (g).

METHODS. The *ntl* probe was described previously¹⁷. *flh* expression was detected as for Fig. 5. The embryos shown in g and h were mounted in glycerol and their genotypes were subsequently confirmed by PCR with primers detecting the presence or absence of the insertion in the *no tail* gene that resulted in the *ntl*^{b195} mutation²³.

Despite the absence of a differentiated notochord, *flh* mutants form a floor plate that appears to be intact in the brain and anterior spinal cord but disrupted more posteriorly. This phenotype, together with the observation that dorsal marginal cells in *flh* mutants transiently express *sonic hedgehog*, suggests that loss of *flh* function partly, but not completely, blocks the ability of axial mesodermal cells to induce floor-plate differentiation. In this respect, it seems that *flh* acts at an earlier step in axial mesoderm development than *ntl*, because floor-plate differentiation is largely unaffected in *ntl* mutants^{8,34}. Further, based on the loss-of-function phenotype, *flh* is required for *ntl* mRNA expression in the developing notochord. However, *ntl* does not simply function downstream of *flh*, as *flh* and *ntl* each require the function of the other for expression in the developing notochord at midgastrulation. This interdependence contrasts with earlier stages, when neither of these genes requires the function of the other for expression. Thus other factors activate *flh* and *ntl* expression in the blastula and early gastrula.

One candidate for an activator of these genes at early stages is *HNF-3β*, which is known from mutational analysis in mouse to be essential for notochord formation^{20,21}. *HNF-3β* has been proposed to act upstream of *Brachyury* in axial mesoderm development, as loss of *HNF-3β* function results in a complete absence of *Brachyury* mRNA in the developing notochord^{20,21}. Further experiments are needed to determine whether *HNF-3β* regulates *flh/Xnot* expression. However, a phenotypic comparison suggests that *HNF-3β* functions at an earlier step in axial mesoderm development than *flh*, because *HNF-3β*[−] mice completely lack floor plate and, presumably, midline mesodermal signalling^{20,21}. Isolation and characterization of more mutations that disrupt notochord development will lead to the identification of additional genes that might regulate *flh* expression in the notochord precursors early in gastrulation. □

tial function in notochord development: *flh* mutants lack a differentiated notochord, and muscle occupies the midline of these animals instead. One interpretation that accounts for these observations is that notochord precursors differentiate as muscle rather than as notochord in the absence of *flh*⁺ function. Recent work strongly supports this possibility: fate map analysis demonstrates that dorsal marginal cells in *flh* mutants form muscle, rather than notochord, as they do in wild-type animals (A.E.M., R. M. Warga and C.B.K., manuscript in preparation). In addition, *flh*[−] cells differentiate as muscle rather than notochord when transplanted to the dorsal region of a wild-type host embryo⁴⁴. Our finding that *flh* expression in the early gastrula is restricted to the embryonic shield, where the notochord precursors reside³ (A.E.M., R. M. Warga and C.B.K., manuscript in preparation), is consistent with a role for this gene in the regulation of notochord precursor cell fate.

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LETTERS TO NATURE

Dynamical evidence for a black hole in the eclipsing X-ray nova GRO J1655 – 40

Charles D. Bailyn*, Jerome A. Orosz*,
Jeffrey E. McClintock† & Ronald A. Remillard‡

* Department of Astronomy, Yale University, PO Box 208101,
New Haven, Connecticut 06520, USA

† Harvard-Smithsonian Center for Astrophysics, 60 Garden Street,
Cambridge, Massachusetts 02138, USA

‡ MIT Center for Space Research, Rm 37-595, Cambridge,
Massachusetts 02139, USA

X-RAY novae are binary systems in which a compact object accretes gas from a companion star. In several cases there is good evidence that the compact object is a black hole^{1–5}. The best evidence for the presence of a black hole comes from measuring the orbital velocity of the companion star and thereby determining the minimum mass of the compact object; when this mass exceeds the maximum mass for a neutron star (≤ 3 solar masses⁶), a black hole seems the only remaining possibility. The unusual X-ray nova GRO J1655 – 40 (refs 7–10) is unique because it emits superluminal radio jets, suggesting that it is a low-luminosity counterpart to active galactic nuclei^{7,9}, which are thought to be powered by accretion onto a massive black hole. Here we report observations of the optical counterpart¹⁰ of GRO J1655 – 40, which show that the system undergoes periodic eclipses; the edge-on geometry thus implied allows a determination of the companion star's true velocity. The mass of the compact object derived from the velocity curve is at least 3.16 ± 0.15 solar masses, which strongly supports its identification as a black hole.

We made photometric measurements of GRO J1655 – 40 on 18–25 March and 5–24 April 1995 with the 0.9-m telescope and CCD imager at the Cerro Tololo Interamerican Observatory (CTIO), and on 28 March to 2 April 1995 with the CTIO 1.5-m telescope and CCD imager (all dates are given in UT). The magnitude of the source was in the range of $16.2 \leq V \leq 16.9$ during this time, about a magnitude brighter than the quiescent value of 17.3 (ref. 7). Spectra were obtained with the Ritchey-Chretien spectrograph on the CTIO 4-m telescope on the nights of 30 April and 2–4 May 1995. On 3 May 1995, during our spectroscopy run, M. Postman obtained five photometric measurements of the source with the CTIO 1.5-m telescope.

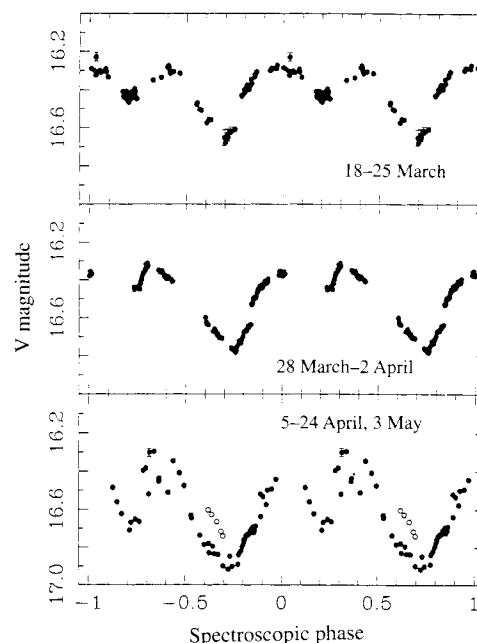


FIG. 1 Lightcurves of GRO J1655 – 40, folded on a period of 2.612 days. The depth of the eclipses is seen to be increasing and approaching the pre-outburst magnitude level of $V = 17.3$. Open symbols on the bottom plot are data from 3 May. Error bars smaller than the symbols have been suppressed.

As the photometric data were obtained, it became clear that the lightcurve showed eclipses, as previous data had suggested¹⁰, and was periodic on a timescale of ≤ 3 days. Unfortunately, only one compelling fiducial point fell within our observing windows, namely a sharp minimum on 2 April (UT) at heliocentric Julian day (HJD) $2,449,809.70 \pm 0.02$. A variety of different periods near 2.6 days could be derived by invoking night-to-night variations of ≤ 0.1 magnitudes in the overall brightness of the source, which are common in such sources during decay from outburst^{11,12}. However the overall lightcurve shape was similar for all possible periods, consisting of a broad triangular primary minimum, and a shorter secondary minimum displaced by about 0.5 in phase from the primary minimum. This lightcurve is similar to that of the eclipsing X-ray binary CAL87 (ref. 13). Cycle-

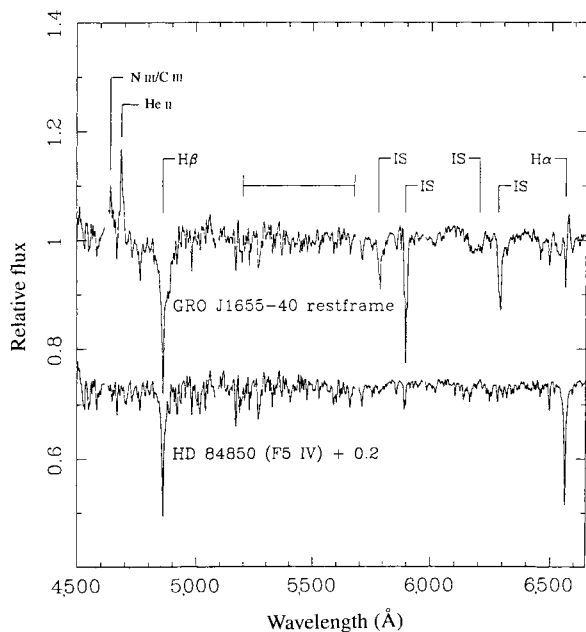


FIG. 2 Top spectrum is the sum of all our data of GRO J1655–40 after each individual spectrum was shifted to the rest frame. Bottom spectrum is of our radial velocity template, HD84850, a F5 subgiant. The combination of the Loral 1,000 × 3,000 pixel detector and the KPGL3 grating gave a wavelength range of 3,850–7,150 Å and a pixel scale of 1.1 Å per pixel. Both spectra have been normalized to a polynomial fit to the continuum. To facilitate comparison with the GRO J1655–40 spectrum, a constant has been added to the template spectrum, so that the absorption lines are of similar depth. The horizontal bar indicates the cross-correlation region, which avoids emission lines, interstellar lines (marked 'IS'), and bad columns of the CCD detector.

to-cycle variations in lightcurve shape as well as a gradual increase in the depth of the primary minimum were superposed on this basic lightcurve shape (Fig. 1).

It seems likely that an eclipse of a large accretion disk by the secondary star is responsible for the primary minimum, and an eclipse of the secondary star by the disk is responsible for the secondary minimum. This conclusion is strengthened by the fact that the source became redder during primary minimum and bluer during secondary minimum, as would be expected given a hot disk and a cooler star. The simultaneous spectroscopic and photometric data obtained on 3 May also strongly support this interpretation (see below). Furthermore, the fraction of the light contributed by the secondary star decreased during the secondary minimum. The implication that the system is close to edge-on is supported by models of the radio jets (ref. 10), which predicts an orbital inclination of $i=85^\circ$. Such edge-on systems allow the use of the powerful tool of eclipse mapping¹⁴, in which a detailed picture of the disk can be built up by modelling the lightcurve. Although the combination of incomplete data on any one orbit and clear orbit-to-orbit variability makes such detailed modelling difficult with our current data, the application of this technique to GRO J1655–40 promises to greatly increase our understanding of accretion processes and jet production near black holes.

The spectra of GRO J1655–40 had less prominent Balmer emission lines and stronger high-excitation lines (for example, He II 4,686 Å and N III/C III 4,640–4,650 Å) than X-ray novae in quiescence¹³, presumably because the outburst was still underway. Nevertheless, it was immediately apparent from the individual spectra that a stellar F-type absorption spectrum was present (see Fig. 2). We used the FXCOR routine in the software package IRAF to cross-correlate the spectra of GRO J1655–40 against that of HD84850, a sixth magnitude F5 subgiant of known radial velocity. The range of wavelengths over which the

cross-correlation was performed is also shown in Fig. 2. We obtained highly significant cross-correlation peaks and radial velocities from each of our 73 spectra, with r -values¹⁵ of at least 7 in all cases. Cross-correlating with other template spectra and wavelength regions yielded similar results.

We fitted a sine curve to the velocity data from 30 April and 3 and 4 May using standard χ^2 minimization techniques. The best-fit sinusoid had a period of 2.601 ± 0.027 days, an epoch of maximum radial velocity of HJD 2,449,839.083 $\pm$ 0.003 and a semi-amplitude $K = 227.2 \pm 3.5$ km s⁻¹ (Fig. 3). The observed period is compatible with the 3 ± 0.2 day periodicity derived from the wiggles seen in the radio jets⁹. Because we had four nine-hour-long spans of data over five nights, aliases of this period are ruled out. Cross-correlating our 34 radial velocity templates with each other reveals discrepancies of up to 40 km s⁻¹ between our observations and the velocities listed in the SIMBAD data base. Therefore we have averaged results from all of our templates to determine the systemic velocity $\gamma = -150 \pm 19$ km s⁻¹, where the error is dominated by systematic effects. Correcting for the Sun's motion and differential galactic rotation results in a radial velocity of -114 ± 19 km s⁻¹ for this object¹⁶. Unfortunately there are currently only upper limits on the proper motion of the source¹⁰.

The large value of γ is unexpected: previous dynamically confirmed black-hole candidates have much smaller values of γ (refs 1–4). Neutron-star systems often have large peculiar velocities, presumably imparted by kicks during an asymmetric collapse, but black holes are not expected to show such behaviour. A detailed consideration of the implications of the high γ value of GRO J1655–40 is given in ref. 16. The most satisfactory explanation seems to be that the black hole in this system did not form as a result of a prompt collapse, but via an intermediate neutron-star stage.

The period (P) and semi-amplitude (K) of the velocity curve result in the following value for the mass function:

$$f(m) = \frac{M_1^3 \sin^3(i)}{(M_1 + M_2)^2} = \frac{PK^3}{2\pi G} = 3.16 \pm 0.15 M_\odot$$

where M_1 is the mass of the compact object, M_2 is the mass of the secondary star, i is the inclination of the orbit to the line of sight, and $M_\odot$ is the mass of the Sun. An inspection of the above equation reveals that the mass function represents the minimum possible mass of the compact object. The maximum stable mass

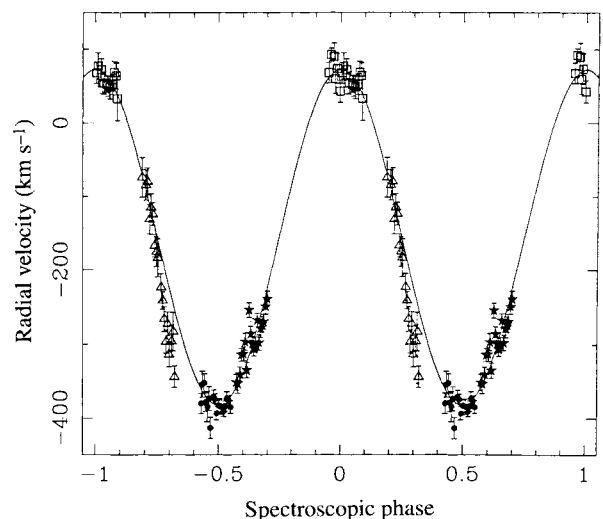


FIG. 3 Radial velocity curve of GRO J1655–40. Filled circles indicate data taken on 30 April; triangles, stars and squares indicate data from 2, 3 and 4 May respectively. Exposure times were typically 1,200 seconds. Errors are derived from fitting a gaussian function to the cross-correlation peak.

of a neutron star is $3M_{\odot}$ for all causal equations of state⁵, and below $2.5M_{\odot}$ for most plausible equations of state¹⁷. Thus, in the absence of exotic matter and/or non-einsteinian gravity, a mass function above $3M_{\odot}$ requires the compact object to be a black hole.

The emission lines varied in shape from night to night, which made it impossible to determine a velocity curve for the accretion disk. We were, however, able to study the characteristics of the secondary star by creating a summed spectrum in its rest frame. We also observed a number of bright stars of known spectral type. We determined limits on the spectral type of the secondary star by multiplying these reference spectra by a constant and subtracting them from the summed rest-frame spectrum of GRO J1655–40. Subtracting spectra of stars of type F3–F6 from GRO J1655–40 resulted in significantly smoother residuals than other types, and we conclude that the spectral type of the secondary star is within this range (Fig. 2). We were unable to determine a luminosity type in this way, but we note that a secondary star that fills its Roche lobe in a 2.6-day orbit must be somewhat larger than a mid-F main-sequence star. In the likely case that the secondary is no more massive than a main-sequence star of equivalent spectral type ($M_2 \leq 1.5M_{\odot}$) and $i \geq 80^\circ$, the compact object would have a maximum mass of $5.4 \pm 0.2M_{\odot}$. The fraction of light from the secondary star was found to be $\sim 40\%$, except during the night of 2 May, when it was $\sim 30\%$. No change in spectral type was observed during our observations.

The steep slope of the velocity measurements on 2 May is incompatible with any sine curve which fits the other three nights of data. As we rotated the spectrograph slit from time to time each night to follow the parallactic angle¹⁸, we do not believe that a steady monotonic drift in our equipment (for example, in the placement of the star in the slit) is likely. This fact, combined with the smooth progression of the 2 May velocity data, suggests that the deviation from the predicted sinusoid is real. These deviations probably do not represent orbital motion of the secondary star, so we did not include the 2 May data in our determination of the parameters of the sinusoidal fit. These data are, however, shown in Fig. 3 for completeness.

Interestingly, the data deviate from the theoretical curve starting at approximately the predicted time of the middle of the secondary eclipse. This is a phase when a number of effects might come into play. First, X-ray heating, which is known to affect radial velocities¹⁹, is at a maximum. However, the lack of changes in the spectral type of the secondary star indicates that this effect must be much smaller in GRO J1655–40 than in Hercules X-1 (ref. 17). Alternatively, the eclipse of the secondary star by the accretion disk might mask part of the secondary star, resulting in an asymmetric contribution from the star's rotational velocity to the observed radial velocity. Although our moderate-resolution spectra do not significantly constrain the rotational velocity of the secondary star, the equatorial rotational velocity of a Roche lobe filling star in a 2.6-day orbit should be $\geq 100 \text{ km s}^{-1}$, significantly larger than the size of the velocity deviations. As the fraction of light contributed by the secondary star was demonstrably lower on 2 May than on other nights, such an effect is plausible.

We attempted to improve our spectroscopic ephemeris by including constraints imposed by the photometry. The photometric data taken on 3 May between spectroscopic phase 0.6 and 0.7 clearly show an ingress into a primary eclipse, implying that primary eclipse is at spectroscopic phase 0.75, as would be expected for an eclipse of the accretion disk. Applying this assumption to the primary minimum observed on 2 April leads to an orbital period of 2.612 ± 0.003 days. As can be seen in Fig. 1, this period results in apparent phase offsets in the photometry obtained between 18 and 25 March. Indeed, no single period fits all the data; periods that align the top two panels of Fig. 1 result in sizeable misalignments with the spectroscopic phase. Given the phase problems with the photometric data, we caution against taking the 2.612-day period at face value. As the source

was brighter during 18–25 March epoch, we suggest that activity in the disk, such as hotspots^{20,21} and superhumps^{11,22}, may be responsible for the observed phase offset. Similarly, we note that the eclipse event observed in August 1994 (ref. 10) also cannot be aligned with our current data, so once again it appears that some combination of X-ray heating, hot spots and superhumps is distorting the lightcurve. When an orbital period sufficiently accurate to trace back to the beginning of the outburst becomes available, these issues may be resolvable. $\square$

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Heterogeneous catalysts obtained by grafting metallocene complexes onto mesoporous silica

Thomas Maschmeyer, Fernando Rey, Gopinathan Sankar & John Meurig Thomas*

Davy-Faraday Research Laboratory,
The Royal Institution of Great Britain, 21 Albemarle Street,
London W1X 4BS, UK

THE synthesis of mesoporous siliceous solids with large-diameter channel apertures (25–100 Å) has greatly expanded the capabilities of heterogeneous catalysis^{1–7}. The large apertures in such mesoporous silicas can, for example, be modified by framework substitution to create highly selective catalysts^{4,5}. Here we show that direct grafting of an organometallic complex onto the inner walls of mesoporous silica MCM-41 (ref. 1) generates a shape-selective catalyst with a large concentration of accessible, well spaced and structurally well defined active sites. Specifically, attachment of a titanocene-derived catalyst precursor to the pore walls of MCM-41 produces a catalyst for the epoxidation of cyclohexene and more bulky cyclic alkenes.

There is currently great interest in titanium-containing zeolitic catalysts for selective oxidations. Titanium ions incorporated into the framework sites of silicalite I and II (that is, TS1 and TS2 respectively, introduced by the Enichem Company)⁸ as well

* To whom correspondence should be addressed.

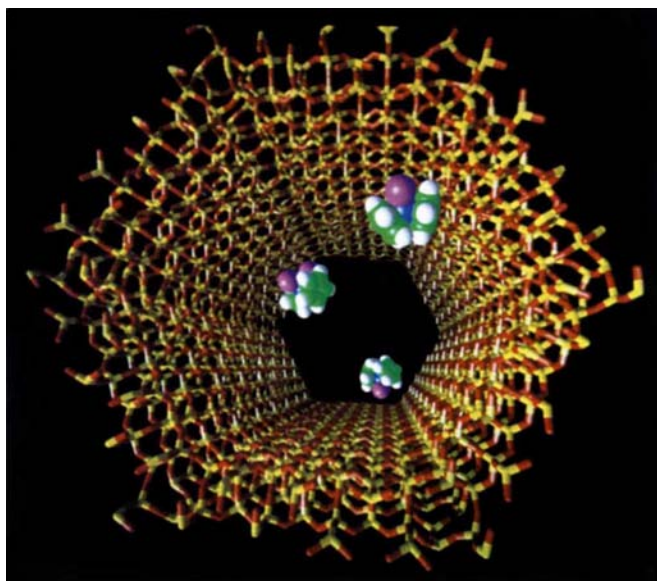


FIG. 1 Computer-generated illustration of the accommodation (diffusion/adsorption) of molecules of titanocene dichloride inside a pore (30-Å diameter) of siliceous MCM-41. For simplicity, none of the pendant $\equiv\text{Si}-\text{OH}$ (silanol) groups, that make it possible to graft organo-metallic moieties inside the mesoporous host, are shown. Si (yellow); O (red); C (green); H (white); Cl (purple); Ti (blue).

as into the framework sites of ZSM-12⁹, ZSM-48^{10,11}, zeolite β ¹² and analogous microporous aluminosilicates (and silico-) phosphates such as ALPO-5, ALPO-11 and SAPO-5 all show remarkable catalytic properties^{13,14}. But one disadvantage of these titanomicro-porous catalysts is that their pore dimensions are too small to allow access to bulky reactants of the kind that dominate most of the chemical transformations which are of central importance in the fine-chemical and pharmaceutical industries. A significant step forward came recently^{4,5} with the preparation and use of Ti-MCM-41 in which the Ti is incorporated (during synthesis) in the framework of mesoporous SiO_2 having a pore diameter of ~ 30 Å. These structured (MCM-41) silicas offer great advantages over silica gels because the former can be shape-selective, intrinsically the latter can never be.

Notwithstanding this improvement, the catalytic performance of the framework-containing Ti-MCM-41 leaves much to be desired. Thus, the turnover frequencies of Ti-MCM-41 for the conversion of small molecules are distinctly inferior to those of TS-1⁸ and Ti-zeolite β ^{5,12,15}, ostensibly because access by the reactants to the Ti active centres is still somewhat limited as the latter are buried within the inner walls of the mesoporous solid.

By extending the concepts of what have come to be known as interfacial coordination chemistry¹⁶, surface organometallic chemistry^{17,18}, and shape-selective catalysis, we have circumvented the above-mentioned difficulties associated with Ti-MCM-41 by grafting titanocene onto the totally accessible inner surfaces of siliceous MCM-41 as shown schematically in Figs 1 and 2. The resulting material, with well separated, well defined, high surface concentrations of Ti-containing active sites, exhibits high catalytic performance.

Titanocene dichloride is superior to TiCl_4 or $\text{Ti}(\text{OR})_4$ as a grafting reagent because, with either of the latter, there is a marked tendency for oligomeric titano-oxo species and/or some anatase by-products to be formed during the grafting. This is circumvented by our method as the relatively stable cyclopentadienyl ligands protect the titanium centre and, hence, prevent either dimerization and/or oligomerization. Moreover, with TiCl_4 , the quantities of evolved HCl are potentially damaging to the siliceous MCM-41, whereas using our methodology the

evolved HCl is scavenged by an amine thereby rendering it harmless.

Titanocene dichloride was dissolved in chloroform and allowed to diffuse into MCM-41 for 30 minutes. The now red MCM-41 was exposed *in situ* to triethylamine to activate the surface silanols of the MCM-41. The colour of the suspension changed from red via orange to yellow over a period of two hours, signifying that the well established substitution of the chloride with alkoxide/siloxide ligands had occurred¹⁹. After extensive washing with chloroform, the organic components (typically 12.7 wt%) of this material were removed by calcination under dry oxygen leaving the white, powdery mesoporous catalyst (Si/Ti = 10, that is, 6.76 wt% Ti), the structural integrity of which had remained intact as established by X-ray diffraction.

All the intermediate materials produced in the catalyst preparation, as well as the catalyst itself during the course of epoxidation and subsequent regeneration, were examined by Ti K-edge X-ray absorption spectroscopy using an *in situ* cell²⁰, the experiments being performed on station 8.1 at the Daresbury Synchrotron Radiation Source (the SRS at Daresbury is operated at 2 GeV with a typical current of 200 mA. Station 8.1 was equipped with a Si(111) monochromator, ion chambers and a 13-element solid-state (Canberra) detector for measurement using transmission and fluorescence mode.

The pre-edge peak (due to a $1s \rightarrow 3d$ transition) was used as a fingerprint in assessing the local structure around the Ti— analogous to procedures used^{21,22} in identifying metal-ion environments of materials containing V and Cr. Here the local structures around the anchored titanocene complex and, after calcination, around the corresponding Ti^{4+} active centre were determined with the aid of both X-ray absorption near-edge structure (XANES) and extended X-ray absorption fine structure (EXAFS).

The pre-edge peak and the EXAFS of the titanocene dichloride adsorbed into MCM-41 were identical to those of the un-anchored starting material, showing that the material is only loosely bound as shown in Fig. 1. In particular, we found identical chlorine and carbon neighbours in both free and MCM-41-adsorbed titanocene dichloride, using rigorous multiple-scattering treatment of the EXAFS data analysis^{23,24}. On reaction with triethylamine, the X-ray absorption spectrum altered significantly. The change in type of neighbours (oxygen for chlorine) and their average bond distance (1.82 compared with 2.33 Å) clearly signify that anchoring had taken place. Moreover, detailed analysis of the EXAFS data disclosed that the two cyclopentadienyl (cp) rings (at ~ 2.39 Å) underwent considerable modification, giving a single ring as opposed to the two in the starting material. The Fourier transform infrared (FTIR) spectrum of this anchored material shows that the $\text{C}=\text{C}$ stretching

TABLE 1 Local structural parameters from Ti K-edge EXAFS

	Scatterer	N	R (Å)	σ^2 (Å ²)
Free $\text{Ti}(\text{Cp})_2\text{Cl}_2$	Cl	2	2.33	0.003
Free $\text{Ti}(\text{Cp})_2\text{Cl}_2$	C	10	2.39	0.005
Adsorbed	Cl	2	2.33	0.003
Adsorbed	C	10	2.39	0.005
Anchored	O	3	1.82	0.008
Anchored	C	5	1.99	0.010
Anchored	Si	3	3.14	0.008
Calcined	O	4	1.81	0.004
Calcined	Si	3	3.30	0.003
Reactive state	O	4	1.84	0.008
Reactive state	O	1	2.25	0.008
Reactive state	O	1	2.40	0.008
Reactive state	Si	3	3.28	0.007

N is the coordination number, R is the distance from the titanium to the scattering atom and σ is the root-mean-square deviation of the interatomic distance about R.

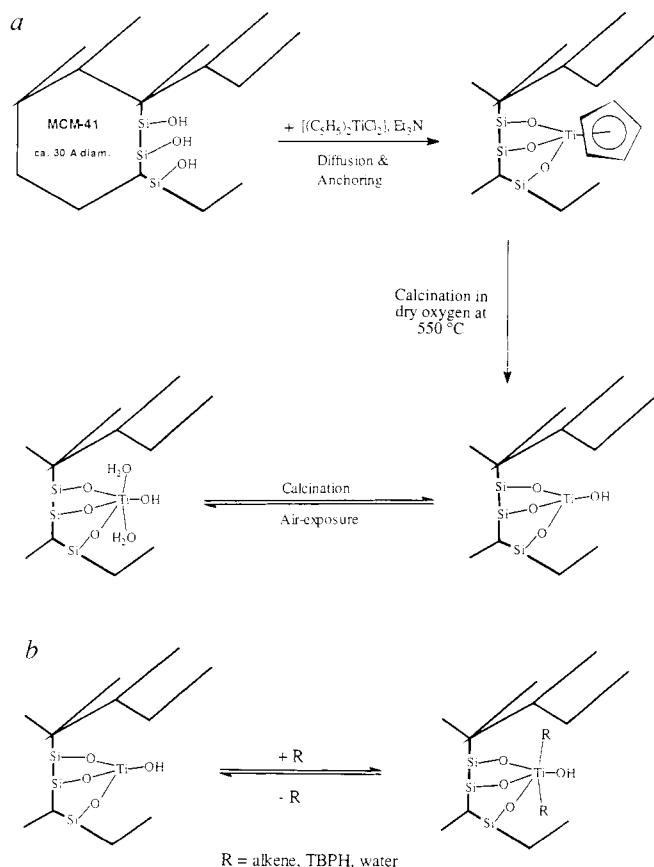


FIG. 2 *a, b*, Schematic representation of the preparation of the grafted catalyst: *a*, the support, anchoring reaction, calcination and reversible reaction of the organometallic-derived Ti-MCM-41 epoxidation catalyst with water on exposure to the atmosphere; *b*, schematic representation of the anchored, active Ti^{4+} species during catalysis.

mode in the region of $1,400\text{ cm}^{-1}$ is strongly altered compared with that of the starting material, thereby corroborating the supposed modifications seen by EXAFS. The bands assigned to the cp ring (appearing at $1,485\text{ cm}^{-1}$ and at $1,365\text{ cm}^{-1}$ in the unanchored material) are shifted by 40 cm^{-1} to higher frequency on anchoring, indicating stronger interaction between the titanium centre and its cp ligand; this is in line with expectation for a shorter Ti–cp distance²⁵. Thus on the basis of EXAFS and FTIR, as well as from chemical considerations, we see that the Ti^{4+} ion is anchored via three oxygens (each linked to silicon) and one cp ring (Fig. 2*a*, top right).

Calcination in oxygen at 550 °C removes the cp ligand (Fig. 2*a*, bottom right) and the resulting material becomes catalytically active. *In situ* measurements by X-ray absorption spectroscopy (XAS) reveal distinct modification in the local structure around the anchored Ti^{4+} ion: there is an increase in pre-edge intensity (Fig. 3*a*) and the Ti–O coordination is close to four in the catalytically active state. (Comparable changes have been reported for Ti incorporated MCM-41^{6,7}, for titanium silicalite, TS-1^{26,27}, and for titanium zeolite β ²⁸; and there is little doubt that four coordinated Ti is the active centre for the selective oxidation of alkenes in the presence of a peroxide.) Further detailed analysis of our data (applying multiple scattering techniques) established the local structure of the active Ti centre. The key features are shown in Fig. 3 (along with the best fit for the EXAFS data and their associated Fourier transforms) and Table 1.

During the course of catalysis, the solid turned to pale yellow (similar to the framework-incorporated Ti-MCM-41 catalyst for

epoxidation reported by us previously^{6,7}). From the significant diminution in intensity of the pre-edge peak (Fig. 3*b*), and other chemical considerations, we processed our raw EXAFS data in terms of distorted six-fold coordination taking into account second-nearest neighbours. The results are summarized in Fig. 3*c–f*.

The catalytic oxidation reactions of cyclohexene and pinene with tetrabutylperoxyhydroxide (TBPH) were performed under argon following standard procedures²⁹. At 40 °C , using a ratio of cyclohexene to TBPH of 1:1.2, and a reactant to catalyst ratio of 1:0.05 by weight using an 8% solution of cyclohexene in chloroform, a maximum turnover frequency of 3 mmol C_6H_{10} per g catalyst per min could be achieved. To our knowledge, this is the highest frequency of turnover reported on epoxidation of alkenes catalysed by Ti-containing mesoporous materials and represents an improvement by about one order of magnitude on previously described preparations⁵. Over a period of 60 minutes, a 50% conversion of cyclohexene with 95% selectivity of TBPH towards epoxidation was recorded. Although the catalyst was essentially deactivated after 90 minutes, we believe that fine-tuning of its preparation and of the reaction conditions should further enhance its remarkable activity. In any event, even without further improvement we have shown that regeneration readily recovers its activity.

In general, the increased performance can be rationalized by noting that (1) all titanium centres are surface species, (2) there

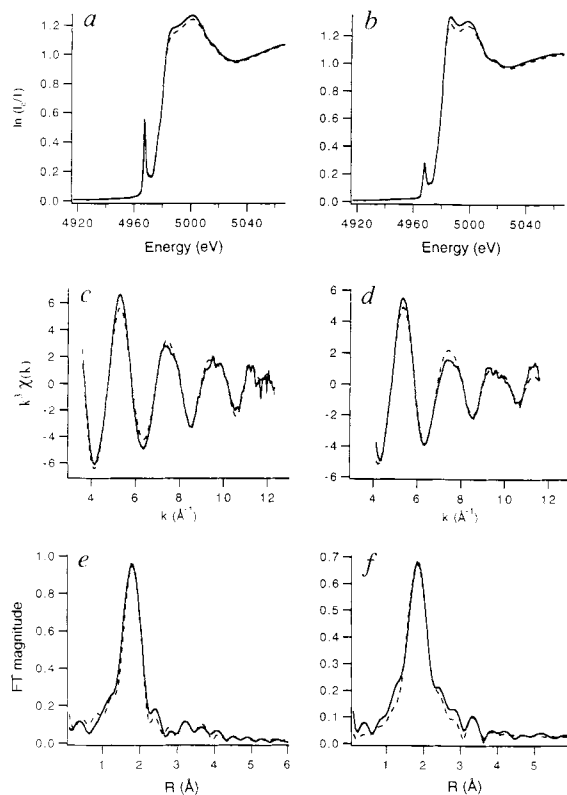


FIG. 3 *a–f*, Summary of the characterization of the catalyst by X-ray absorption spectroscopy (XAS). *a, b*, XANES part of the Ti K-edge XAS data, detailing (solid lines) the pre- and near-edge structures of the calcined catalyst (*a*) and during the course of catalytic reaction (*b*). The dashed lines in *a* and *b* represent the recalcined catalyst after catalytic reaction and the calcined catalyst exposed to atmospheric conditions, respectively. *c–f*, Best fit to the Ti K-edge EXAFS data of the calcined (activated) catalysts (*c*), during catalytic reaction (*d*) and their associated Fourier transforms, (*e* and *f*, respectively). See Table 1 for bond distances. In *c–f*, the solid lines represent the experimental data, whereas the dashed lines are calculated from the model structures contained in Table 1.

are no detectable amounts of oligomeric, and hence inactive, species present, and (3) the titanocene can diffuse thoroughly into the channels before grafting, thereby yielding highly dispersed catalytic sites which, in turn, present maximum access to reactant molecules.

Additionally, and uncommonly for most heterogeneous catalysts, we have quantitatively determined the atomic environment of the active site. Furthermore, this new material is active in catalytic oxidations of bulky reactants (for example, pinene and TBPH gave 20% conversion after 12 hours reacting), whereas microporous zeolitic materials are inactive owing to the narrowness of their pores. Moreover, amorphous silicas as supports have no potential as shape-selective catalysts.

Our catalyst is readily regenerable to its active state by re-calcining the expired form in oxygen at 550 °C. The X-ray diffraction and XAS data, as well as the catalytic characterization of the recycled catalyst, show no detectable structural degradation nor loss in activity of any significance. The XAS data clearly show the re-formation of four-coordinated titanium identical to that of the fresh catalyst (the XANES spectrum is shown as dotted curve in Fig. 3a). On exposure to atmospheric conditions a distorted six-fold coordinated titanium, similar to that of the reacted catalyst, is seen by XAS. (XANES data of the calcined catalyst exposed to atmospheric conditions are shown as a dotted curve in Fig. 3b.) However, this exposure does not deactivate the catalyst.

Our grafting approach should be applicable for other metallogenenes, and indeed we have found already that a very high dispersion of isolated, tetrahedral Mo(VI) centres may be generated on the walls of mesoporous MCM-41. This should allow for fine-tuning of catalytic activity without the need to modify the mesoporous framework itself. Furthermore, comprehensive characterization of these catalysts by EXAFS allows us to track each entire system from the embryonic stage of synthesis (adsorption) through to the active state of the catalyst during operation and after its regeneration. Thus we can establish a direct relationship between structure and function by combining catalytic studies with *in situ* XAS studies. □

A new mechanism for calcium loss in forest-floor soils

Gregory B. Lawrence*, Mark B. David† & Walter C. Shortle‡

* US Geological Survey, 425 Jordan Road, Troy, New York 12180, USA

† University of Illinois, W-503 Turner Hall, 1102 South Goodwin Avenue, Urbana, Illinois 61801, USA

‡ USDA Forest Service, Northeastern Forest Experiment Station, Durham, New Hampshire 03824, USA

CALCIUM is the fifth most abundant element in trees, and is an essential component for wood formation and the maintenance of cell walls. Depletion of Ca from the rooting zone can result in acidification of soil¹ and surface water² and possibly growth decline and dieback of red spruce^{3,4}. During the past six decades, concentrations of root-available Ca (exchangeable and acid-extractable forms) in forest-floor soils have decreased in the northeastern United States^{5,6}. Both net forest growth and acid deposition have been put forth as mechanisms that can account for this Ca depletion^{5,6}. Here, however, we present data collected in red spruce forests in the northeastern United States that are inconsistent with either of these mechanisms. We propose that aluminium, mobilized in the mineral soil by acid deposition, is transported into the forest floor in a reactive form that reduces storage of Ca, and thus its availability for root uptake. This results in potential stress to trees and, by increasing the demand for Ca, also decreases neutralization of drainage waters, thereby leading to acidification of lakes and streams.

Forest-floor soil is formed on top of mineral soil by the accumulation of nutrient-rich organic matter from roots and litter. Thick (>5 cm) forest floors develop in northern temperate and boreal forests, where cool temperatures retard decomposition. Because of their high nutrient content relative to the mineral soil, these soils are the primary rooting layer in forests where they are found. To evaluate mechanisms that cause Ca depletion in forest-floor soil, we collected soil and soil-solution samples in 12 undisturbed red spruce stands, and one stand that has received 1,800 equiv. ha⁻¹ yr⁻¹ of (NH₄)₂SO₄ since November 1989⁷. The stands, located in New York, Vermont, New Hampshire and Maine, were selected to represent the range of environmental conditions and stand health for red spruce in the northeastern United States. Soils were analysed for exchangeable Ca (by NH₄Cl extraction⁸), exchangeable Al and H (by KCl extraction⁹), pyrophosphate-extractable Al⁸, acid-extractable Al (by 1:1 HNO₃ digest¹⁰), and total Al (by neutron activation¹¹). Bulk densities of soils in the Oa horizon (which is part of the forest floor) were estimated with a model developed by Federer¹²; we used the methods of soil-solution collection and analysis described in refs 13 and 14, respectively).

Because net forest growth and acid deposition can involve exchange between adsorbed Ca and H, observed decreases in exchangeable-Ca concentrations in the forest floor were expected to be accompanied by an increase in exchangeable-H concentrations. Results indicated, however, that concentrations of exchangeable Ca in the soil Oa horizon were correlated with neither exchangeable-H concentrations (Fig. 1a) nor with soil pH ($P > 0.1$), but were inversely related to concentrations of exchangeable Al (Fig. 1b). This negative correlation between concentrations of exchangeable Ca and exchangeable Al in the forest floor suggests that forest-floor decreases in Ca may be associated with increases in concentrations of reactive forms of Al rather than H. This contention is supported by results of analysing archived Oa-horizon soil samples collected from red spruce-balsam fir (*Abies balsamea* (L.) Mill) stands at the Hubbard Brook Experimental Forest (HBEF), New Hampshire. These data indicated that concentrations of exchangeable and acid-extractable Al

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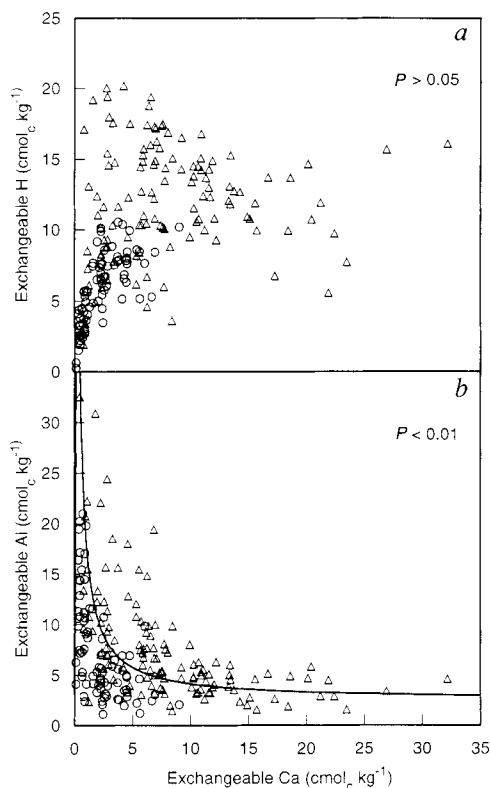


FIG. 1 Concentrations of exchangeable H (a) and Al (b) as a function of exchangeable-Ca concentrations in the Oa horizon of stands of red spruce (triangles) and mixed hardwood (circles); $\text{cmol}_c \text{kg}^{-1}$ indicates cmol of charge per kg soil. Red spruce data are based on analysis of 36 soil samples from each of eight sites, plus 18 samples collected at each of five additional sites. Each triangle represents the value for three soil samples that were combined before analysis. Hardwood data are based on 68 samples collected on Slide Mountain, New York at elevations ranging from 800 to 1,100 m. The curve in b represents the best fit for the model $y = 1.2(1/x) + 4.7$; ($R^2 = 0.41$). The significance probability (P) is listed in each panel.

have increased with a corresponding decrease in Ca concentrations over the past two decades, although the increase for exchangeable Al was not statistically significant (Table 1).

Mineral forms of Al which are physically mixed into the forest floor can be mobilized by naturally derived organic acids, although dissolved Al is typically highly undersaturated with respect to mineral solubility in the forest floor¹⁵. Acid deposition provides an additional source of H^+ that could possibly enhance dissolution of mineral Al; however, our results showed that exchangeable-Al concentrations were unrelated to mineral-Al concentrations, and dissolved inorganic Al concentrations were unrelated to soil-solution pH ($P > 0.10$). Despite the relative abundance of mineral Al in the forest floor (85% of total Al), exchangeable Al concentrations were probably controlled by organically complexed Al. Mineral saturation indices showed that dissolved inorganic Al concentrations in the forest floor were more than 1,000 times lower than would have been observed had any type of mineral known to occur in these soils been the primary control, and exchangeable Al was positively correlated ($P < 0.01$; $R^2 = 0.60$) with reactive-nonexchangeable Al, which consisted of 70% organically complexed Al. In organic soils, control by organically complexed Al, rather than mineral solubility, has often been demonstrated^{15,16} and decreases in pH have been shown to cause decomplexation of Al from solid-phase organic matter¹⁶. The naturally low pH of forest-floor solutions¹⁷, however, indicates that acid deposition has not enhanced dissolution of mineral Al or decomplexation of organic Al within these forest floor soils. Production of organic acids

causes solution pH values at these sites to range from 3.3 to 3.8 (Table 2); these values are below the pH of precipitation in this region which averages about pH 4.4 and is seldom less than 4.0 (ref. 18). There is no established mechanism to explain how the addition of precipitation with a pH above 4.0 could enhance dissolution of mineral Al in forest floors that have solution pH values naturally less than 4.0.

In contrast to the forest floor, soil-solution pH of the B horizon (which is part of mineral soil) is likely to have been lowered by acid deposition at these sites. The pH of mineral-soil solutions were higher than forest-floor solutions (Table 2) because organic acids leached from the forest floor tend to be removed from solution by sorption to mineral surfaces¹⁹. Strong acids derived from acidic deposition, however, are much less easily immobilized and therefore are more effective than organic acids at acidifying soil solutions and mobilizing Al in the mineral soil²⁰. The influence of strong acids on Al mobilization in the B horizon is supported by the positive correlation we observed between solution concentrations of H^+ and inorganic Al ($R^2 = 0.54$; $P < 0.01$), typical for mineral soils receiving acidic deposition²¹.

An alternative mechanism for increased exchangeable-Al concentrations in the forest floor is suggested by a strong positive correlation found between the Al/Ca ratio of soil solution in the B horizon and the exchangeable-Al content of the forest floor (Fig. 2). We propose that Al, mobilized in the mineral soil by acid deposition, is transported into the forest floor where it accumulates in a reactive mostly organically complexed form. Both biocycling and water movement have been established as mechanisms that can transport Al into the forest floor^{22,23}. A recent study showed that biocycling (including Al transported from roots in the mineral soil to roots in the forest floor) plus atmospheric deposition was roughly 60% of forest-floor leaching losses at Tunk Mountain, Maine^{22,24}. Root uptake of Al in the B horizon is likely at all of our study sites because the ratios of inorganic Al to Ca are greater than 1.0 (Table 2), the value above which root exchange sites readily adsorb Al at the expense of Ca (ref. 25). A rising water table provides an additional way to increase the pool of reactive Al in organic surface horizons, as does upward movement of water through rapid drying of the forest floor by evapotranspiration. When mineral-soil solution

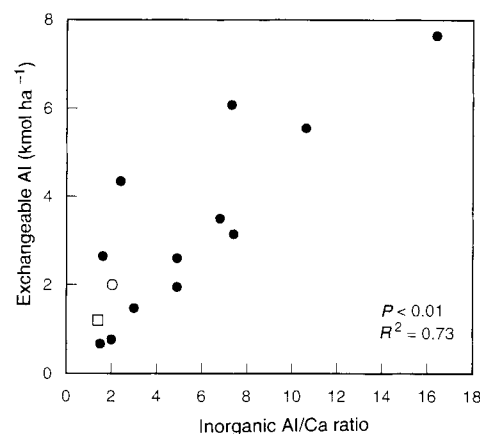


FIG. 2 Exchangeable-Al content of Oa horizons of red spruce stands as a function of the molar concentration ratio of inorganic Al to Ca in B-horizon soil solution. Exchangeable Al was expressed as content to normalize the data for varying forest-floor thickness. Each filled circle represents the mean of 18–36 soil and soil-solution samples (combined into 6–12 samples before analysis) collected at each of 12 sites. One of the 13 sites (Hubbard Brook) was omitted because there was insufficient B-horizon soil to sample. The open circle represents the mean of 68 soil samples and 31 seep-water samples, collected in a mixed hardwood forest on Slide Mountain, New York at elevations ranging from 800 to 1,100 m. The open square represents soil and seep-water concentrations measured at Tunk Mountain, Maine by Rustad²⁴. The significance probability is given by P ; the coefficient of determination is given by R^2 .

TABLE 1 Al and Ca concentrations in Oa-horizon soils

Sampling period	Exchangeable (cmol _c kg ⁻¹)		Acid-extractable (cmol _c kg ⁻¹)	
	Al	Ca	Al	Ca
1969–70	2.5 ^a (1.1)	8.3 ^a (4.4)	19.3 ^a (10.2)	9.9 ^a (6.4)
1987, 1992	3.7 ^a (2.9)	3.5 ^b (2.1)	37.0 ^b (21.6)	4.6 ^b (2.9)

Samples were collected in spruce-fir stands at the Hubbard Brook Experimental Forest, New Hampshire, USA. Samples collected in 1969 and 1970 were averaged together, as were those collected in 1987 and 1992. Values are means based on 9–14 samples. Statistically significant differences ($P < 0.05$) between sampling periods, determined from the Wilcoxon nonparametric test and the Tukey means separation test, are indicated by superscripts with different letters. Standard deviations are given in parentheses.

that is enriched in Al relative to Ca moves into the forest floor, Al is more effectively retained than Ca owing to the high affinity of Al for organic binding sites¹⁹. When this same solution then drains back into the mineral soil, the Al to Ca ratio is decreased. Mulder *et al.*²³ found, in two Norwegian watersheds, Al-enriched organic surface horizons caused by upward movement of water from mineral horizons; simulation with the BROOK90 hydrological model²⁶ indicated that, in HBEF soils, movement of water from the mineral soil to the forest floor at a rate of 1 mm d⁻¹ was common in the summer.

The quantitative importance of exchangeable Al in the forest floor relative to exchangeable Ca is shown by the exchangeable Al to Ca ratios in Table 2. Mobilization of Al in the mineral soil has significantly contributed to the documented decrease of root-available Ca in the forest floor by (1) reducing uptake of Ca from the mineral soil that could be biocycled into the forest floor, (2) providing a supply of reactive Al, that when transported into the forest floor, exchanges with Ca, enabling the latter to be leached, and (3) increasing Al saturation so that the number of exchange sites available for adsorbing added Ca is reduced (because Ca has minimal ability to displace adsorbed Al¹⁹). Increasing Al saturation in the forest floor is particularly significant in relation to the importance of atmospheric Ca as an input to the available pool of Ca. Recent work at a high-elevation red spruce stand indicated that about half of the Ca in the forest floor was of atmospheric origin²⁷, even though atmospheric inputs of basic cations have decreased over the past two decades²⁸. A high degree of Al saturation will limit retention of Ca added from atmospheric deposition, increasing the likelihood that dissolved Ca will pass below the rooting zone before it can be taken up by roots.

Although this study has focused on red spruce forests, evidence of Al transport from the mineral soil to the forest floor can also be shown for hardwood forests. Relations among concentrations of exchangeable Al, Ca and H are similar for red spruce forests and a mixed hardwood forest on Slide Mountain, New York (Fig. 1), as are relations between mineral-soil solution and forest-floor exchangeable Al pools (Fig. 2). Transport of Al from the mineral soil to the forest floor is likely to occur in regions that are receiving acid deposition and have forest soils with well defined organic surface horizons overlying shallow mineral soils with an inherent low base saturation. Ecosystems of this type occur over large regions of eastern North America, Scandinavia and Russia.

By reducing the availability of Ca in the primary rooting zone, increases in reactive forms of Al will potentially slow growth and weaken the stress tolerance of trees. Increased demand for Ca by trees will also limit the neutralization of drainage waters because a greater fraction of available Ca will be retained within the terrestrial ecosystem. Reversing the accumulation of reactive Al in the forest floor by anticipated future decreases in atmospheric deposition of strong acids will be hindered by high Al to Ca ratios in mineral-soil solutions, the tendency for Al to strongly adsorb to organic matter, and continued decreases in atmospheric deposition of Ca. □

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TABLE 2 Soil-solution and soil chemistry in the Oa horizon and upper 10 cm of the B horizon

Site	Oa horizon				Upper B horizon			
	pH	Ca	Inorganic Al	Ex. Al/ex. Ca	pH	Ca	Inorganic Al	Ex. Al/ex. Ca
Sleepers River*, Vermont	3.7	60	<1.5	0.2	4.5	8	13	12
Groton, Vermont	3.3	98	2	0.3	4.8	6	8	5
Howland, Maine	3.3	142	13	0.4	4.5	5	14	22
Kossuth, Maine	3.4	85	9	0.8	4.6	5	11	34
Bartlett, New Hampshire	3.5	61	<1.5	0.5	4.5	2	16	25
Hubbard Brook*, New Hampshire	3.6	38	2	0.8	—	—	—	—
Whiteface Mtn*, New York	3.7	31	13	0.8	4.6	7	20	11
Crawford Notch, New Hampshire	3.3	47	3	1.1	4.4	4	19	27
Bear Brook ref.†, Maine	3.8	30	13	1.9	4.4	8	19	20
Big Moose Lake, New York	3.5	22	2	1.2	4.1	5	30	27
Cone Pond, New Hampshire	3.5	18	7	5.2	4.4	2	18	53
Mt Abraham*, Vermont	3.7	13	26	5.6	4.1	4	25	16
Bear Brook treat.‡	3.7	15	87	7.1	4.1	4.5	74	21

Concentrations of Ca and inorganic monomeric Al are in $\mu\text{mol l}^{-1}$. Units for the ratios of soil exchangeable Al to Ca are $\text{cmol}_c \text{ kg}^{-1}$. Each value represents the mean of 36 samples that were collected individually and then combined into 12 samples for analysis, except for sites followed by an asterisk, for which each value represents 18 samples collected individually and combined into six samples for analysis. Upper B-horizon soil-solution data were not collected at Hubbard Brook because of insufficient mineral soil development.

† Site name refers to the undisturbed Bear Brook watershed.

‡ Site name refers to the treatment Bear Brook watershed that received 1,800 equiv. ha⁻¹ yr⁻¹ of $(\text{NH}_4)_2\text{SO}_4$ since November 1989.

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Simulation of abrupt climate change induced by freshwater input to the North Atlantic Ocean

Syukuro Manabe & Ronald J. Stouffer

Geophysical Fluid Dynamics Laboratory/NOAA, Princeton University, Princeton, New Jersey 08542, USA

TEMPERATURE records from Greenland ice cores^{1,2} suggest that large and abrupt changes of North Atlantic climate occurred frequently during both glacial and postglacial periods; one example is the Younger Dryas cold event. Broecker³ speculated that these changes result from rapid changes in the thermohaline circulation of the Atlantic Ocean, which were caused by the release of large amounts of melt water from continental ice sheets. Here we describe an attempt to explore this intriguing phenomenon using a coupled ocean–atmosphere model. In response to a massive surface flux of fresh water to the northern North Atlantic of the model, the thermohaline circulation weakens abruptly, intensifies and weakens again, followed by a gradual recovery, generating episodes that resemble the abrupt changes of the ocean–atmosphere system recorded in ice and deep-sea cores⁴. The associated change of surface air temperature is particularly large in the northern North Atlantic Ocean and its neighbourhood, but is relatively small in the rest of the world.

The coupled model^{5,6} consists of general circulation models (GCMs) of the atmosphere and oceans and a simple model of land surface that includes the budgets of heat and water. It is a global model with realistic geography. The atmospheric GCM includes the seasonal variation of insolation and predicted cloud cover. It has nine vertical finite-difference levels. The horizontal distributions of predicted variables are represented by spherical harmonics (15 associated Legendre functions for each of 15 Fourier components) and by corresponding grid points. The oceanic GCM uses a finite-difference technique with a regular grid system which has horizontal spacing of 4.5° latitude $\times$ 3.75° longitude and 12 vertical levels.

The control experiment is a 1,000-year time integration of the coupled model⁷ described above. The initial conditions for the control experiment have realistic seasonal and geographical distributions of surface temperature, surface salinity and sea ice, with which both the atmospheric and oceanic model states are nearly in equilibrium. When the time integration of the model starts from this initial condition, the model climate drifts towards its own equilibrium state which differs from the realistic initial condition described above. To reduce this drift, the fluxes of heat and water obtained from the atmospheric component of the coupled model are modified by given amounts before they

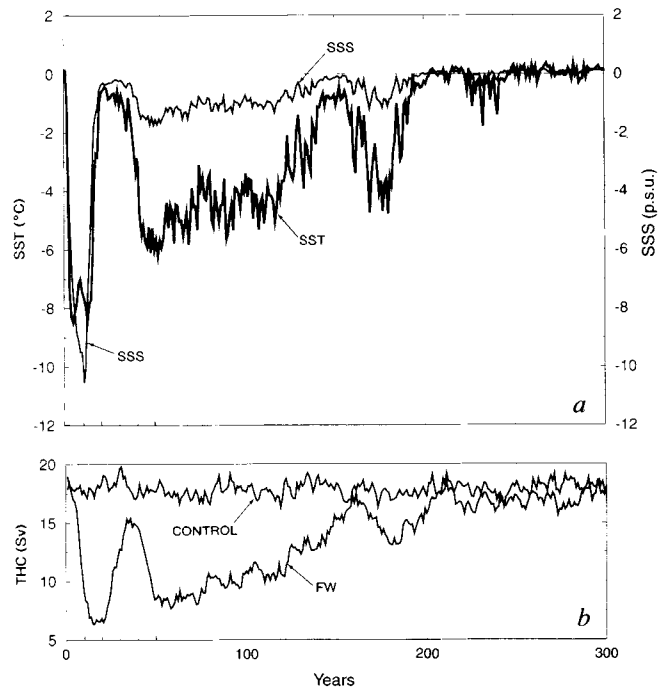


FIG. 1 a, Time series of the deviations of sea surface temperature (SST) and sea surface salinity (SSS; p.s.u., practical salinity units) from their initial values (that is, 7°C and 35 p.s.u., respectively) at a grid point in the Denmark Strait (60.75°N , 37.50°W) obtained from the FW experiment. b, Temporal variations of the rate of THC in the North Atlantic obtained from the control and FW integrations. Here, the rate of THC is defined as the maximum value of the stream function of meridional circulation in the North Atlantic (Fig. 4).

are imposed at the oceanic surface. Because the adjustments are determined before the time integration of the coupled model, and are not correlated to the transient surface anomalies of temperature and salinity which can develop during the integration, they are unlikely to either systematically amplify or damp the anomalies. The adjustments do not eliminate the shortcomings of the model dynamics which could distort the simulated transients⁸. But the adjustments do prevent the rapid drift of the model state from the realistic initial condition, which could seriously distort the results of a numerical experiment. The identical adjustments are also applied to the freshwater (FW) experiment described below.

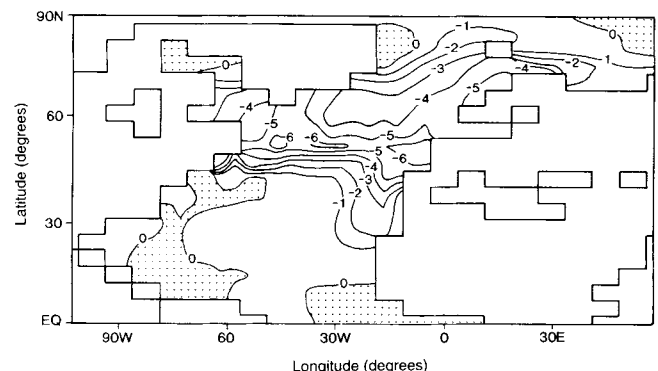


FIG. 2 Horizontal distribution of SSS anomalies (in units of p.s.u.) averaged over the 10-year period from year 11 to year 20 of the FW experiment. The areas of positive anomalies are shaded. Here, the anomaly represents the difference between the 10-year mean state of the FW integration and the 100-year (years 501–600) mean state of the control integration.

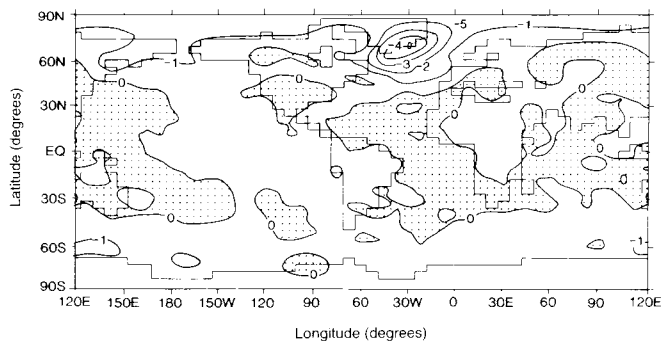


FIG. 3 Horizontal distribution of surface air temperature anomaly ($^{\circ}\text{C}$) averaged over the 10-year period from year 51 to year 60 of the FW experiment. The areas of positive anomalies are shaded. Here, the anomaly represents the difference between the 10-year mean state of the FW integration and the 100-year (years 501–600) mean state of the control integration.

In the FW experiment conducted here, a surface freshwater flux of 1 Sv ($10^6 \text{ m}^3 \text{ s}^{-1}$) is released uniformly over the latitude belt between ~ 50 and 70° in the North Atlantic Ocean during the first 10-yr period. Although the freshwater flux is turned off at the end of the tenth year, the time integration is performed over a period of 300 years. (Fairbanks⁹ estimated that the rate of the pre-Younger Dryas discharge of melt water into the entire world oceans peaked at 0.44 Sv , which is smaller than the rate of 1 Sv used here. However, the total amount of discharge is much larger than that imposed in the present experiment.) The initial condition for the FW experiment is the state of the cou-

pled ocean–atmosphere model at year 501 of the 1,000-year control integration. The change of the model state induced by the freshwater flux is examined by comparing the FW and control integrations. We note that the melt water is applied to the simulated state of the current interglacial rather than the last glacial period. It is therefore desirable to reassess the response of the thermohaline circulation (THC) in a glacial environment when the glacial states of oceans and the atmosphere have been successfully simulated and the rates of freshwater input into the North Atlantic are better known.

Because of the infusion of fresh water described above, sea surface salinity (SSS) in the Denmark Strait is reduced abruptly during the decade of the water infusion, followed by a rapid recovery as indicated in Fig. 1a. By the second decade, the region of large negative SSS anomalies extends to the west of the north African coast and Arctic Ocean (Fig. 2). The reduction of surface salinity during the first decade of the experiment is accompanied by a very rapid lowering of sea surface temperature (SST) centred around the Denmark Strait by as much as 8°C followed by an almost abrupt warming and cooling (Fig. 1). By the sixth decade, the region of negative SST and surface air temperature anomalies extends from 40°N in the Atlantic to most of Greenland, Scandinavia and the west European coast (Fig. 3). During the next few hundred years, both SST and SSS increase and recover their normal values, with substantial fluctuations around years 120 and 200 (Fig. 1).

The sudden drop of SST during the first decade of the FW experiment results partly from the reduction of convective activity which mixes the cold surface water with the warmer subsurface water of the ocean. The SST drop is also attributable to the rapid weakening of the thermohaline circulation (Fig. 1b), which advects warm surface water towards the northern North Atlan-

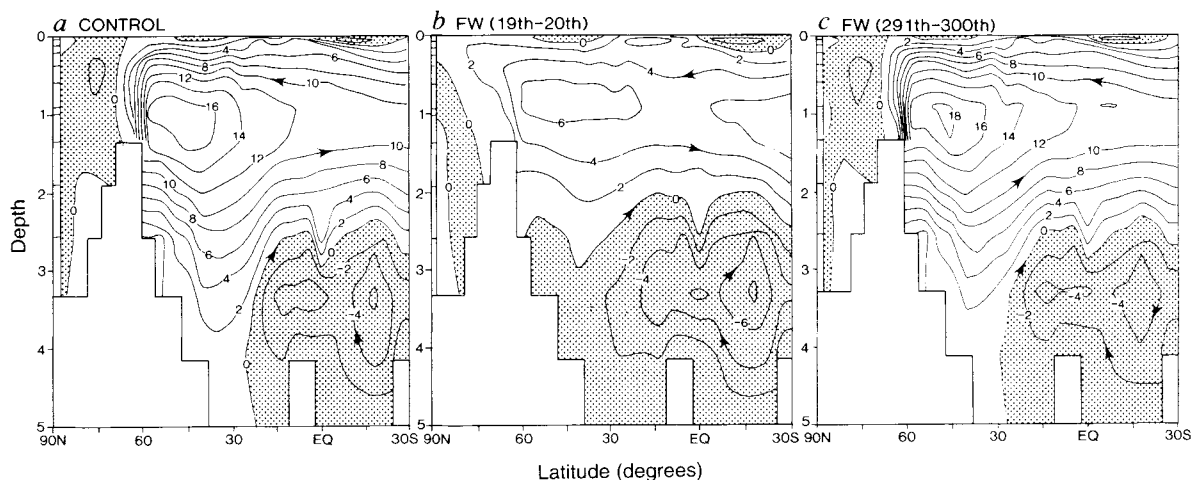


FIG. 4 Latitude–depth distribution of the stream function which represents the meridional overturning in the North Atlantic in units of sverdrups ($10^6 \text{ m}^3 \text{ s}^{-1}$), averaged over: a, years 501–600 of the control experiment; b, years 19 and 20 and c, years 291–300 of the FW experiment. (Depth is given in kilometres; areas of clockwise circulation are shaded.) During the few initial decades, the THC in the North Atlantic markedly weakens and becomes shallower (compare a and b), allowing stronger deep inflow of Antarctic bottom water. The initial capping of the Atlantic Ocean by the low-density, fresh surface water chokes off the heat exchange between the atmosphere and ocean and raises the temperature of subsurface water, rapidly weakening the THC during the first decade of the experiment. At the end of the second decade, the THC extends poleward of 65°N in the near-surface layer (b). The associated extension of the northward advection of warm surface water is responsible for the rapid increase of SST and SSS in the Greenland Sea during the second decade (Fig. 1a). The northward extension of warm advection disappears by year 40 of the experiment, causing the reduction of SST and SSS during the fourth decade (Fig. 1a). It appears that the northward extension (into the Norwegian/Greenland seas) occurs

because the density of surface water is too low to sink at normal latitudes (that is, around 60°N). The gradual recovery and eventual restoration (c) of the overturning intensity, which follows the rapid initial fluctuation, is attributable partly to the vertical subsurface water column of relatively high density which is restored in the sinking region of the THC by ~ 70 years of the experiment. The increase of the water column density is due not only to the horizontal spreading and disappearance of the very fresh surface water but also to the reduced supply of warm water with relatively low density from the south into the sinking region of the THC. The gradual reintensification of the THC is also attributable to the reduction of subsurface water density in low latitudes which is due to the weakened upwelling of dense water associated with the large initial weakening of the THC. The increase in the meridional gradient of density due to the density changes of opposite sign in the sinking and rising regions of the THC results in the increase of not only the meridional but also the zonal density gradient²⁰ in the upper layer of the ocean, intensifying the meridional circulation and causing the gradual recovery in the intensity of the THC.

tic. The large initial weakening and reintensification of the THC appear to be induced by the massive, initial infusion of fresh, low density water and its abrupt termination, respectively. After the initial fluctuation, the THC reintensifies gradually (Fig. 1b), increasing both SST and SSS during the next few hundred years. The damped oscillation of the THC, which occurs during the reintensification, resembles the weaker oscillation which Delworth *et al.*¹⁰ found in the control experiment. The structures of the THC at the beginning, shortly after the termination of the freshwater infusion, and at the end of the experiment are illustrated and discussed in Fig. 4, and its legend.

The coupled model produced large and rapid changes in surface air temperature and the rate of deep-water formation reminiscent of those inferred from the palaeoceanographic^{4,11} and ice-core records^{1,2}. But during the glacial and postglacial periods, one can identify many cold episodes of low ice-core $\delta^{18}\text{O}$ which last much longer than the simulated period of low SST in the present FW experiment. Obviously, the cold period could last longer if a meltwater flux of smaller magnitude were applied much longer than 10 years, or a massive armada of icebergs was discharged and melted in the middle of the cold period¹². Both of these mechanisms, which release the fresh water with low $\delta^{18}\text{O}$, could have operated during the cold event of 14,500 ^{14}C -years ago, accounting for the $\delta^{18}\text{O}$ minimum in planktonic foraminifera noted by Keigwin and Lehman¹³. On the other hand, the total amount of melt water infused around the beginning of the Younger Dryas event might have been substantially larger than that imposed in the present experiment⁹. If so, the THC could be weakened enough to keep it at a reduced intensity despite the relatively small supply of fresh water during most of the cold Younger Dryas period. It is likely that the rate of meltwater supply during the Younger Dryas was too small to reverse the positive $\delta^{18}\text{O}$ anomaly associated with low SST, yielding the planktonic $\delta^{18}\text{O}$ maximum noted by Keigwin and Lehman¹³.

By use of a coupled ocean-atmosphere model, Manabe and Stouffer¹⁴ obtained two stable equilibria, that is, active and inactive modes of the THC in the Atlantic Ocean. They suggested that the inactive mode resembles the oceanic state of Younger Dryas. However, the palaeoceanographic evidence¹⁵ indicates not only a markedly reduced deep-water formation but also a significant ventilation of the upper ocean layer during the cold period. It is therefore likely that the transient state of weak and shallow THC encountered during the present FW experiment is more consistent with the palaeoceanographic signatures of Younger Dryas than the inactive equilibrium mentioned above. The freshwater-induced transitions among multiple equilibria of a simple coupled model were examined, for example, by Stocker *et al.*¹⁶ and Rahmstorf²¹.

The evolutions of the THC and SST, which were recently obtained by Rahmstorf¹⁷ by use of his coupled model with a highly simplified atmosphere and idealized geography, and without the seasonal variation, is quite different from those described in the present study. His simulation shows that, in response to an infusion of fresh water, the THC rapidly weakens but restores its original intensity very quickly, leaving behind an equilibrium state of shallow convection and cold surface water. In the coupled model used here, it is not possible to sustain such shallow convection indefinitely at a given grid point, because it is influenced by the noisy and seasonally varying atmosphere.

The recent study of Manabe and Stouffer^{18,19} reveals that, associated with the CO_2 -induced warming of the model atmosphere, the poleward transport of water vapour increases, causing marked increases in precipitation, and accordingly, freshwater supply in the high latitudes. The simulated multi-century response of the THC to the doubling of atmospheric CO_2 (refs 18, 19) resembles the response to the freshwater input described here. Thus substantial changes in the intensity and distribution of the THC could also occur in response to future increases of greenhouse gases in the atmosphere. □

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Plate boundary reorganization at a large-offset, rapidly propagating rift

R. N. Hey*, P. D. Johnson*, F. Martinez*, J. Korenaga†, M. L. Somers‡, Q. J. Huggett‡, T. P. LeBas‡, R. I. Rusby‡ & D. F. Naar§

* Hawaii Institute of Geophysics and Planetology, School of Ocean and Earth Science and Technology, University of Hawaii, Honolulu, Hawaii 96822, USA

† MIT/WHOI Joint Program, Woods Hole Oceanographic Institution, Woods Hole, Massachusetts 02543, USA

‡ Institute of Oceanographic Sciences, Wormley, Godalming GU8 5UB, UK

§ Department of Marine Science, University of South Florida, St Petersburg, Florida 33701, USA

THE existence of rapidly spinning microplates along the southern East Pacific Rise has been documented by geophysical swath-mapping surveys^{1–6}, and their evolution has been successfully described by an edge-driven kinematic model⁷. But the mechanism by which such microplates originate remains unknown. Proposed mechanisms^{1–10} have generally involved rift propagation¹¹, possibly driven by hotspots or changes in direction of sea-floor spreading. Here we present geophysical data collected over the Earth's fastest spreading centre, the Pacific–Nazca ridge between the Easter and Juan Fernandez microplates (Fig. 1), which reveal a large-offset propagating rift presently reorganizing the plate boundary geometry. A recent episode of rapid 'duelling' propagation of the historically failing spreading centre in this system has created a 120×120 km overlap zone between dual active spreading centres, which may be the initial stage of formation of a new microplate.

The dual spreading centres, shown by shallow ridges and acoustically reflective sea floor, overlap between about 28.5° – 29.5° S, 113° – 112° W (Figs 2, 3). The West ridge has been propagating south^{3,12} for ~ 1.5 Myr, transferring young Pacific lithosphere to the Nazca plate (shown by the rotated fabric extending northeast from the overlap zone). It is the shallowest part of the entire East Pacific Rise, reaching above 2,100 m. It is also the most highly inflated, defined by cross-sectional area above the average 0.5 Myr depth³⁰, reaching nearly 9 km². This propagator has created a shallow V-shaped area bounded by pseudofaults¹¹ which indicate a steady propagation rate nearly equal to the spreading rate, ~ 135 km Myr⁻¹. The characteristic parabolic propagator tip results from the acceleration from no spreading to the full rate^{13,14}. Spreading along most of the West ridge

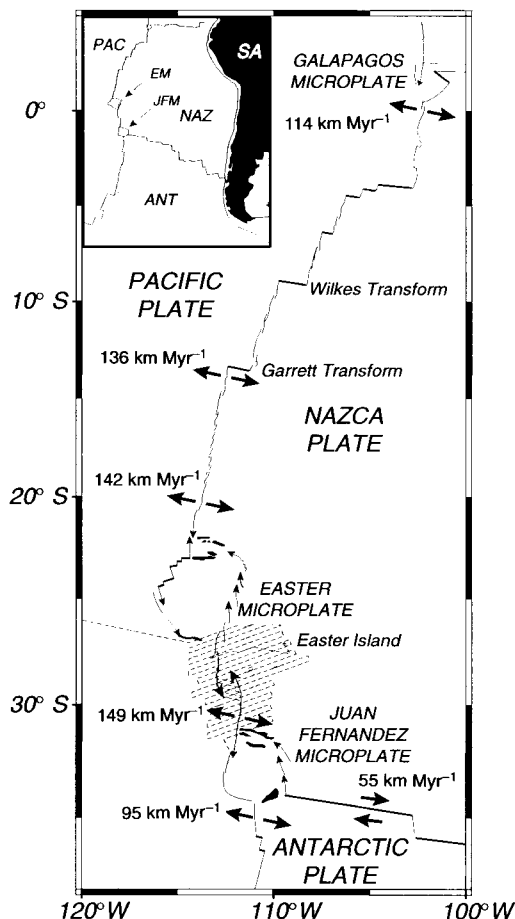


FIG. 1 Location map modified from refs 6 and 28. Light lines are ridges, those with arrows are propagating. Heavy straight lines are transform faults. Spreading rates are from ref. 16, modified by the revised age of 0.78 Myr for the Brunhes/Matuyama reversal¹⁷.

segments has been highly asymmetric, with recent accretion rates on segment W2 ~ 5 times higher to the east than west (Fig. 3), suggesting multiple unresolved rapid propagation events^{13,15}. The recent spreading rate here is significantly lower than the 150 km Myr^{-1} observed on the eastern system and predicted¹⁶ for Pacific–Nazca opening (these rates use the revised astronomically calibrated timescale¹⁷ which gives rates $\sim 6\%$ slower than recently thought). This crustal deficit was mostly taken up by spreading on the failing rifts during episodes of duelling propagation.

The East ridge away from the tip is also shallow and unusually flat ($2,350 \pm 20 \text{ m}$) despite large variations in inflation. Although it has been the failing rift in this propagating rift system, a narrow V-shaped area of highly reflective shallow sea floor cutting through older less-reflective, transferred lithosphere (Fig. 2b) indicates that it has propagated north recently¹⁸, creating an $\sim 15,000 \text{ km}^2$ overlap zone. Duelling propagation has been recognized elsewhere^{19–22} on much smaller scales and attributed to competing magma sources^{20,21}.

The 1:1 aspect ratio of the overlap zone is similar to those of the Easter^{1–3} and Juan Fernandez^{4–6} microplates and large propagator systems²³ rather than the 3:1 ratio characteristic of overlapping spreading centres^{20,21}. The southern boundary of this zone is marked by a narrow linear ridge (Fig. 2a) trending $\sim 110^\circ$; no localized boundary structure is seen in the north. This boundary does not at present intersect the East ridge axis but ends at what we interpret as an inner pseudofault produced by East ridge propagation $\sim 0.2 \text{ Myr}$ ago, indicating a minimum

northward propagation velocity of $\sim 600 \text{ km Myr}^{-1}$. Most of the overlap zone is characterized by northeast-trending structures similar to the oblique structures found in the Galapagos 95.5° W propagator shear zone and interpreted there as resulting from ‘bookshelf’ faulting²³. Fault plane solutions (Fig. 3) for this area are consistent with bookshelf faulting²⁴, as is shipboard three-component magnetometer vector analysis²⁵. Most of the earthquakes occur in the inner pseudofault zones of the duelling propagators. The southeastern part of the overlap zone shows much lower reflectivity than the rest (Fig. 2b), indicating a piece of older Pacific lithosphere transferred into this zone by the southward propagation of the West rift. This area is also relatively shallow, suggesting that lithospheric transfer here involves compression or possibly seamount formation.

Despite the preponderance of northeast-trending structures in the overlap zone, there are two northwest-trending structures near the southern margin (Figs 2, 3). The deep ($\sim 3,500 \text{ m}$) graben trending $\sim 135^\circ$ near 29.5° S , 112.5° W resembles rift structures mapped in Afar (P. Tapponnier and I. Manighetti, personal communication) and is probably a current axis of tectonic rifting with slow spreading (D. McKenzie, personal communication). The narrow scarp just east of this graben forms the southwestern margin of the old lithosphere in the overlap zone and extends into the southern boundary zone. Because of the age asymmetry across this scarp, it is different from the structures identified as failed rifts.

The failed rifts northeast of this area are evident in the side-scan reflectivity and structural data (Figs 2, 3) as relatively young areas of high reflectivity and northwest-trending structures cutting into the relatively older zone of transferred lithosphere (fossil overlap zone). The failed rift tips line up along a trend of $\sim 033^\circ$, in good agreement with the 029° trend predicted by theoretical equations^{14,26} for rift propagation. Some of these failed rifts are deep grabens, typical of several propagator systems²⁶. Others are bathymetric highs which more closely resemble the abandoned ridges on the 20.8° S duelling propagator system²⁰.

There appear to have been about six previous major failed rifts left behind on the Nazca plate, with the (now) northward-propagating East rift segment E1 destined to be the next in this series (Fig. 3) unless a new microplate forms here. Although they penetrate $50\text{--}100 \text{ km}$ into the zone of transferred lithosphere, the rift widths are typically less than 10 km , with the widest (other than E1) $\sim 30 \text{ km}$ wide. Although spreading must slow on these features as they fail, which probably explains why many are grabens²⁶, this still suggests very short spreading histories at these locations. The general lack of major changes in trend of the transferred sea-floor fabric across the failed rifts is evidence that they failed rapidly following episodes of ‘superfast’ propagation or ridge jumps, very similar to those modelled as cyclical ridge jumps in the Juan de Fuca area²². The spacing of the failed rifts suggests an irregular episodicity of $\sim 0.3 \text{ Myr}$. The series of failed rifts appears to be bounded to the northeast by the western part of the SOEST fracture zone (Figs 2, 3), although some rifts seem to have propagated slightly north of this fracture zone just before failing.

Figure 4 shows the hypothesized schematic evolution of this area from a stable dextral Pacific–Nazca transform fault south of the Easter microplate, to a giant propagator system with episodic duelling propagation of the failing rift, to its present configuration. At $\sim 1.5 \text{ Myr}$ the dominant southward propagation began, as well as contemporaneous northward propagation^{1,3,12} away from this same shallow area. The change in trend of the SOEST fracture zone suggests a clockwise rotation of Pacific–Nazca motion at that time. These propagating segments (shown in Fig. 4 by the diamond-shaped pseudofault pattern) are the shallowest of the entire East Pacific Rise system, suggesting that they are probably driven both to north and south by increased magma supply^{12,13} (probably from the nearby Easter hotspot) and the resulting gravitational spreading

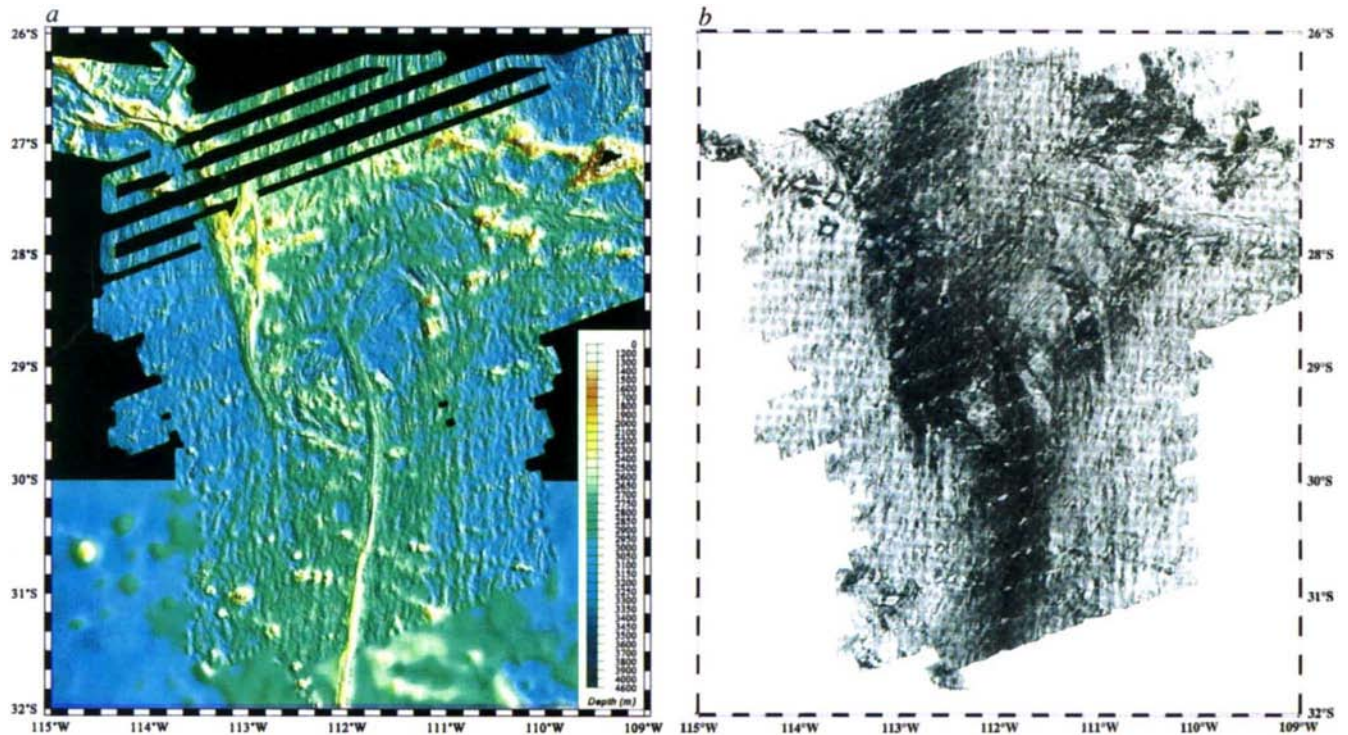


FIG. 2 *a*, Colour-shaded bathymetric relief map, illuminated from the northeast. High-resolution 10-km-wide SeaBeam 2000 bathymetric swaths are overlaid onto the wider (24 km) GLORI-B swaths. This was the first expedition in which this modified GLORIA system collected swath bathymetry. The southwestern margin of the Easter microplate is digitized from hand-contoured SeaMARC II data³. The regional data

south of 30° S are derived from satellite altimetry data²⁹. *b*, GLORI-B sidescan sonar mosaic. Strong acoustic backscatter returns (usually associated with recent volcanism or rough surfaces) are shown dark, weak backscatter areas (usually associated with sedimented areas or shadow zones) are white. Data have been digitally processed to correct for geometrical distortions and to equalize the gain at different ranges.

stresses²⁷. The propagation is facilitated by increased driving forces and decreased resistive forces²⁷ at these 'superfast' spreading rates. All ridges presently spreading faster than 142 km Myr^{-1} (Fig. 1) are being reorganized by rift propagation²⁸.

Is this overlapping system a microplate or a propagator? The clear history of failed rift tips suggests the latter. Also, the NE structural trends within the overlap zone (Figs 2, 3) are consistent with the maximum rotation of 42° predicted by the propagating rift equations^{14,26}. If the overlap zone were behaving as a

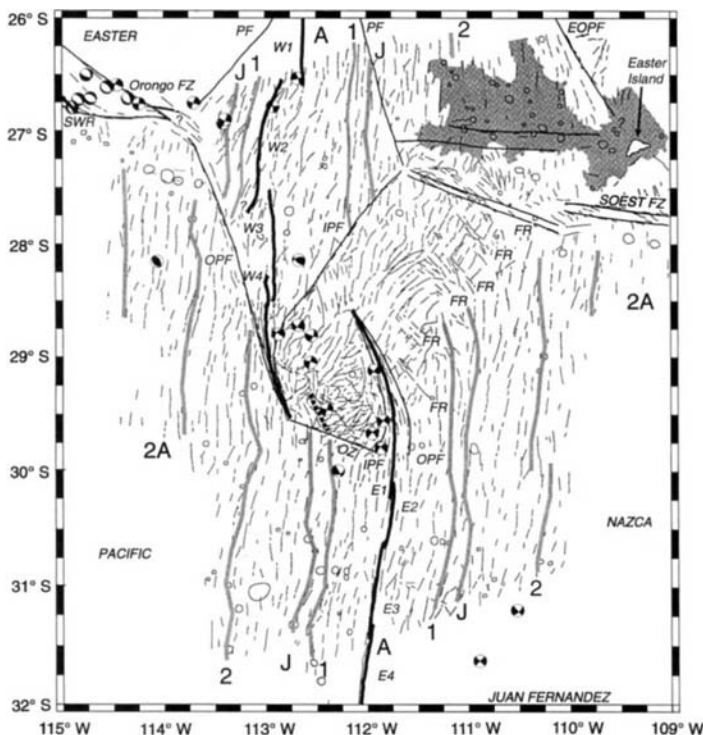


FIG. 3 Simplified tectonic interpretation based primarily on GLORI-B sidescan sonar (Fig. 2*b*) and SeaBeam 2000 bathymetry (see Fig. 2*a*). Heavy lines show active plate boundaries, the heavy dashed line is a new rift graben, medium lines are pseudofaults (PF) and fracture zones (FZ), and light lines are representative fault scarps showing sea-floor structural fabric. The zone of lithosphere transferred from the Pacific to the Nazca plate is marked by northeast-trending fabric extending northeast from the 29° S overlap zone. Cross-cutting structures in this zone are failed rifts (FR), and OZ is the southern boundary of the overlap zone. Circular structures are volcanoes, the shaded area (top right) is the Ahu volcanic field, and beachballs are focal mechanisms (from the Harvard centroid moment tensor catalogue on the Internet (<http://tempo.harvard.edu/CMT.html>), and from ref. 24 for locations near the overlap zone). Lightly shaded lines are isochrons from magnetic anomalies (1 is Brunhes/Matuyama reversal, J is Jaramillo event). SWR is the Southwest rift of the Easter microplate and E1–E4 and W1–W4 are the East and West segments of the inter-microplate ridge system. OPF and IPF are the outer and inner pseudofaults of the duelling propagators, EOPF is the outer pseudofault of the initial Easter microplate propagator.

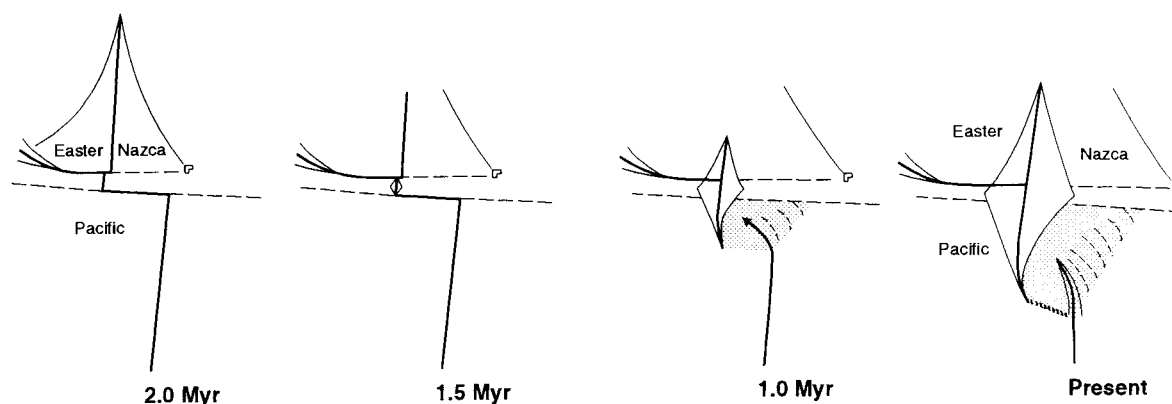


FIG. 4 Schematic evolution from transform fault to duelling propagators to possible proto-microplate. Heavy lines are active plate boundaries, long dashed lines are fracture zones, short dashed lines are failed rifts. Light lines are V-shaped pseudofault wakes of dominant propagators, arrows show briefly active duelling propagators. The northward propagator with outer pseudofault extending to Easter Island, shown

schematically at 2 Myr (from ref. 3), is the one which seems to have initiated Easter microplate formation¹⁻³, probably driven by the magma pulse that formed Easter Island. Shading shows transferred lithosphere. Details of this evolution will be presented elsewhere (J.K. and R.N.H., manuscript in preparation).

microplate, the 120-km diameter requires⁷ a rotation rate of 72° per Myr to match the regional spreading rates. The close fit of observed and predicted structures based on the propagating rift equations thus indicates at most a very brief period of additional microplate rotation. However, there are notable differences between the fossil and present overlap zones. No analogue of the NNW-trending rift graben, the northwest-trending scarp to the east, or the transform-like southern boundary in the present overlap zone is observed in the fossil overlap zone. This suggests that the tectonic behaviour has changed recently. One possibility is that a new microplate may be beginning to form here. A small rift graben adjacent to a transform boundary is also seen within the proposed Wilkes "nannoplate"³¹, although grabens in migrating extensional relay zones have also been proposed to be characteristic of propagating rift systems³².

Although there may have been a recent brief attempt to begin to rotate as a mostly rigid microplate, the successful evolution of this duelling propagator system into an edge-driven microplate⁷ would require the eventual formation of a northern boundary as well. The width of this overlap zone (~120 km) is slightly smaller than those of the pervasively deformed cores of the Easter² and Juan Fernandez⁵ microplates (~130–140 km), suggesting that this is about the scale at which pervasive sea-floor shearing of the overlap zone is no longer possible, the lithosphere stops deforming and begins to rotate as a rigid microplate^{3,6,7,9,18}. The aspect ratios of the deformed cores of the microplates are ~3:1 (Easter) and ~2:1 (Juan Fernandez), suggesting that for this overlap zone to evolve into a similar microplate there would have to be a linkage event resulting from very rapid propagation that would double or triple the overlap length. □

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Elasticity of forsterite to 16 GPa and the composition of the upper mantle

Thomas S. Duffy*, Chang-sheng Zha, Robert T. Downs, Ho-kwang Mao & Russell J. Hemley

Geophysical Laboratory and Center for High-Pressure Research, Carnegie Institution of Washington, 5251 Broad Branch Road, NW, Washington DC 20015, USA

NEARLY 60 years ago, Bernal¹ proposed that a polymorphic phase transformation in olivine might be responsible for the seismic velocity discontinuity near 410 km depth in the mantle. Phase equilibria experiments^{2,3} have since shown that the olivine (α) to wadsleyite (β) transition in $(\text{Mg,Fe})_2\text{SiO}_4$ occurs at the appropriate pressure (13.8 GPa) under mantle conditions. Comparison of laboratory measurements of the acoustic velocity contrast in the α - β system to the magnitude of the seismically observed discontinuity at 410 km provides a way to constrain the olivine content of the mantle at this depth. Here we report measurements of the

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* Present address: Consortium for Advanced Radiation Sources, The University of Chicago, 5640 South Ellis, Chicago, Illinois 60637, USA

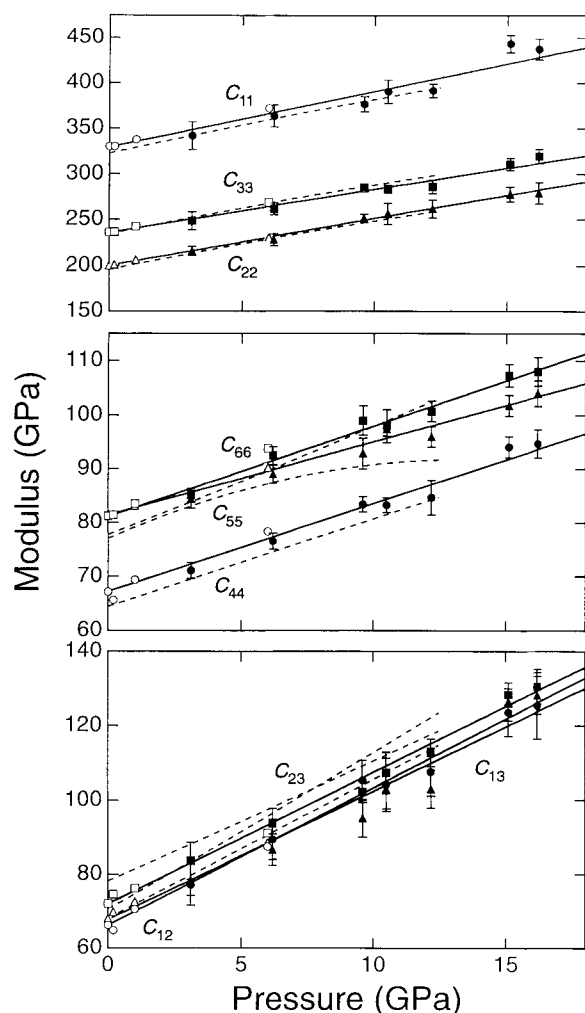


FIG. 1 Elastic moduli of single-crystal forsterite at 295 K as a function of pressure. Solid symbols with error bars (2σ) are present data. The root-mean-square misfit between the measured velocities and those calculated from the best-fitting elastic moduli is typically 0.6%. Additional scatter in the elastic moduli may arise from errors in measurement of pressure and scattering angle, correlations between fitted parameters, and non-hydrostatic stresses. Solid lines are least-squares fits to present results combined with ambient-pressure data¹². The dashed lines are fits to ISS data¹⁷ for $(\text{Mg}_{0.9}\text{Fe}_{0.1})_2\text{SiO}_4$. Open symbols show results of ultrasonic studies of Mg_2SiO_4 at the highest pressure attained in each experiment^{4,5,15}.

full set of elastic moduli of single-crystal forsterite ($\alpha\text{-Mg}_2\text{SiO}_4$) at pressures between 3 and 16 GPa, using Brillouin scattering in a diamond anvil cell. At 13.8 GPa, the aggregate compressional- and shear-wave velocities of $\alpha\text{-Mg}_2\text{SiO}_4$ are $2.7 \pm 0.7\%$ lower than predicted from earlier low-pressure data^{4,5}. From our data, and assuming a homogeneous mantle composition, the seismic velocity contrast at 410 km depth can be satisfied only by a mantle containing less than $\sim 40\%$ olivine. This is well below the olivine abundance assumed in peridotite-based upper-mantle models.

The pyrolite model² of mantle composition is derived from the complementary melting relationships of basalts and peridotites, and contains $\sim 60\%$ olivine by volume. The primary uncertainty associated with this model is that it is derived from samples that originate from shallow depths (≤ 200 km) and assumes chemical homogeneity of the mantle as a whole. Compressional- (P) and shear-wave (S) seismic velocity profiles⁶ provide the most direct constraints on the deep mantle. A number of studies⁷⁻¹¹ have addressed whether seismic data and laboratory measurements of acoustic wave velocities in $(\text{Mg,Fe})_2\text{SiO}_4$ are

consistent with a pyrolite mantle at 410 km depth, but have reached conflicting conclusions, in large part because of uncertainties in the relevant pressure and temperature dependences of the elastic moduli. Here our new measurements of the elasticity of $\alpha\text{-Mg}_2\text{SiO}_4$ to transition-zone pressures are combined with other recent elasticity data^{7,12-18} on the α - β system to constrain the amount of $(\text{Mg,Fe})_2\text{SiO}_4$ consistent with laboratory and seismic data for the 410 km discontinuity.

Single crystals of synthetic forsterite were polished to flat plates ~ 20 – 60 μm thick in a plane intersecting the three crystal axes at nearly equal angles. Electron microprobe, polarizing microscopy, and X-ray diffraction confirmed that the samples were pure, unstrained single-crystal Mg_2SiO_4 . The samples were loaded with ruby and a pressure-transmitting medium into a diamond anvil cell with 95° conical openings¹⁹. Pressures were determined by ruby fluorescence²⁰. An argon pressure-transmitting medium was used at 3.1 and 6.1 GPa, a 4:1 methanol-ethanol mixture was used at 9.6 and 12.2 GPa, and helium was used at 10.5, 15.1 and 16.2 GPa, where the α -phase persists metastably at room temperature. Additional spectra were recorded at 9.6 GPa with a sample polished in the b - c plane. X-ray diffraction was carried out at each pressure to determine the crystal orientation and density, and to obtain information about the sample stress state²¹.

Using Brillouin scattering, acoustic velocities can be determined from the frequency shift of light scattered from thermally generated acoustic waves. Brillouin spectra were recorded using an Ar^+ laser and a tandem Fabry–Perot interferometer²². The velocities of compressional and two shear acoustic waves were measured as a function of orientation at 10° intervals in a single plane at each pressure. The nine second-order elastic moduli of an orthorhombic crystal are related to acoustic velocities through Christoffel's equation²³. The acoustic velocities were inverted using nonlinear least squares to obtain the elastic moduli as well as the three eulerian angles that specify the crystal orientation. The individual moduli were determined to a precision of 1–3% (1σ) and the crystal orientation is determined to $\pm 2^\circ$. The orientation obtained from the Brillouin data is consistent with that determined by X-ray diffraction. Because of the lack of pure mode propagation directions, correlation coefficients between pairs of moduli could be significant. Monte Carlo simulations demonstrate that correlation errors largely cancel when computing aggregate properties.

The elastic moduli of forsterite at pressures between 3.1 and 16.2 GPa are shown in Fig. 1. All elastic moduli vary linearly with pressure within the resolution of our data. A recent study¹⁷ used impulsive stimulated scattering (ISS) to measure the elastic moduli of $(\text{Mg}_{0.9}\text{Fe}_{0.1})_2\text{SiO}_4$ to 12.5 GPa in the diamond cell. The differences between our results and that study can be plausibly attributed to differences in sample iron contents. The most significant difference is that we find that C_{55} remains linear to 16.2 GPa in contrast to the nonlinear behaviour reported in ref. 17. The reason for this is not understood at present, but has no effect on the main conclusions of this study.

To date, models for upper-mantle mineralogy⁸⁻¹¹ have used very-low-pressure ultrasonic data^{4,5} (to ≤ 1 GPa) extrapolated to higher pressures for comparison to seismic data. The pressure derivatives of the moduli determined here are uniformly lower

TABLE 1 Elastic properties of $(\text{Mg}_{0.9}\text{Fe}_{0.1})_2\text{SiO}_4$

Property	α -phase	β -phase
K_S (GPa)	129 (1)*	174 (1)†
G (GPa)	79 (1)*	110 (1)†
$(\partial K_S/\partial P)_T$	4.2 (2)	4.8 (2)‡
$(\partial G/\partial P)_T$	1.4 (1)	1.7 (1)‡
$(\partial K_S/\partial T)_P$ (GPa K^{-1})	−0.017 (1)*	−0.018 (3)§
$(\partial G/\partial T)_P$ (GPa K^{-1})	−0.014 (1)*	−0.014 to −0.024

* Refs 12, 14; † ref. 26; ‡ ref. 7; § refs 16, 18.

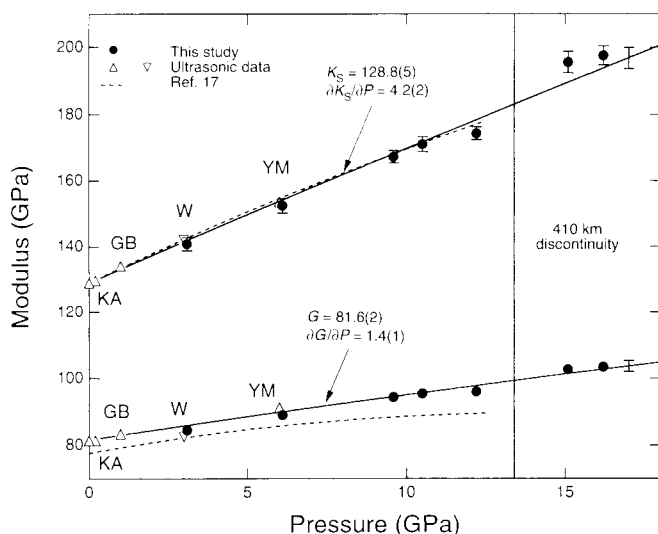


FIG. 2 Aggregate bulk and shear moduli of forsterite as a function of pressure. The solid symbols are data of this study, and solid lines are obtained from third-order finite strain fits to the data. Error bars represent the Voigt and Reuss limits²⁴ of the calculated aggregate moduli and are approximately the size of the symbols for the shear modulus. Confidence limits (1σ) on the fitted curve are shown by the error bar at 17 GPa. Dashed lines are from ISS data¹⁷ for $(\text{Mg}_{0.9}\text{Fe}_{0.1})_2\text{SiO}_4$. Open symbols are from single-crystal ultrasonic studies of forsterite, with symbols shown at the highest pressure achieved in each experiment (KA⁴, GB⁵, YM¹⁵). The inverted triangle (labelled W) shows 3-GPa ultrasonic results¹³ for $(\text{Mg}_{0.9}\text{Fe}_{0.1})_2\text{SiO}_4$. Vertical line shows the pressure of the 410 km seismic discontinuity.

than those of the low-pressure data by $30 \pm 10\%$ (ref. 4) and $20 \pm 10\%$ (ref. 5). Systematic errors in, for example, pressure calibration are a possible explanation for the higher pressure derivatives measured in those studies.

The aggregate bulk modulus (K_S) and shear modulus (G) were calculated at each pressure from the average of the Voigt and Reuss bounds, which are limiting values of the moduli of a random, polycrystalline aggregate obtainable from single-crystal elasticity data²⁴ (Fig. 2). Fitting the data to finite strain expressions²⁵ yields values of 4.2 ± 0.2 and 1.4 ± 0.1 for the pressure derivatives of the adiabatic bulk and shear modulus, respectively (Table 1). The bulk and shear moduli, together with the density, define the compressional- and shear-wave velocities of a random polycrystalline aggregate. At 13.8 GPa, the aggregate velocities measured here are $2.7 \pm 0.7\%$ below those predicted from extrapolation of the previous data at 1 GPa or lower^{4,5}. Our results are in good agreement, however, with recent forsterite data¹⁵ to 6 GPa. For the bulk modulus, our results agree with data on Fe-bearing samples to 3 GPa (ref. 13) and 12.5 GPa (ref. 17). The pressure derivative of the bulk modulus determined from the Brillouin data is consistent with that determined solely from X-ray diffraction²¹.

At ambient pressure and temperature, the P- and S-wave velocity contrasts between the α - and β -phases of Mg_2SiO_4 are²⁶ $\sim 13\%$. One-dimensional seismic velocity profiles of the upper mantle constructed from analysis of body waves at both long and short periods yield velocity contrasts near 410 km that are generally in the range of 4–5% for compressional waves and 4–4.6% for shear waves^{6,27–29}. The amount of forsterite consistent with the magnitude of the discontinuity is only 30–40% by volume, if the α - β velocity contrast is independent of pressure and temperature.

We next consider how the application of pressure affects the velocity contrast between the two phases. Ultrasonic measurements of acoustic velocities in a polycrystalline aggregate of β - Mg_2SiO_4 to 3 GPa have recently been reported⁷. Using pressure derivatives obtained from those measurements (Table 1)

together with 1 GPa ultrasonic data for the α -phase⁴, it was previously concluded that the α - β velocity contrast decreases significantly with pressure (at room temperature)⁷. But when our new values for the elasticity of the α -phase are used in this analysis, the velocity contrast is found to be nearly unchanged from ambient values (12% for P waves and 14% for S waves) at 13.8 GPa. Thus, the velocity contrast between the α - and β -phases of Mg_2SiO_4 is largely independent of pressure at ambient temperature.

We must then consider the combined effects of pressure and temperature on the α - β velocity contrast. This is done using a procedure outlined previously¹⁰ and the parameters of Table 1. The densities and moduli are corrected for the effect of temperature and extrapolated adiabatically using third-order finite strain theory²⁵. The elastic moduli are taken to be linear functions of temperature^{12,14} and are corrected for the effect of 10 mol% Fe.

Recently, new measurements of the temperature sensitivity of the elastic moduli of the α -phase^{12,14} and the isothermal bulk modulus of the β -phase^{16,18}, have been reported. These indicate that the temperature sensitivity of the bulk modulus is similar across the phase transition. The only parameter which has not been measured is the temperature dependence of the shear modulus, $\partial G/\partial T$, for the β -phase. We consider two possible values of this parameter. Measured values of $\partial G/\partial T$ for 15 silicates³⁰ fall in a range of -0.08 to $-0.014 \text{ GPa K}^{-1}$. We adopt $\partial G/\partial T = -0.014 \text{ GPa K}^{-1}$ as a plausible estimate of $\partial G/\partial T$ for β - $(\text{Mg}_{0.9}\text{Fe}_{0.1})_2\text{SiO}_4$, identical to its value for the α -phase. Of the $\partial G/\partial T$ values reported for ~ 80 alkali halides, oxides and silicates³⁰, only a few are larger than -0.02 GPa K^{-1} in magnitude, and the largest reported value is $-0.024 \text{ GPa K}^{-1}$ (for MgO). We adopt this value as an upper-bound estimate of $\partial G/\partial T$ for β - Mg_2SiO_4 .

Sound velocities in the $(\text{Mg,Fe})_2\text{SiO}_4$ system along a 1,250 °C adiabat are shown in Fig. 3, together with seismic velocity profiles. The acoustic velocity jump in the α - β system far exceeds that observed in the seismic data when $\partial G/\partial T$ is constant across the transition. The fraction of olivine corresponding to the magnitude of the seismic discontinuity is 27% for shear waves, and 32% for compressional waves, assuming seismic velocity discontinuities of 4.3% and 4.5%, respectively. Although subject to potential systematic biases, the velocity jump in bulk sound velocity can be estimated by differencing P- and S-wave velocity

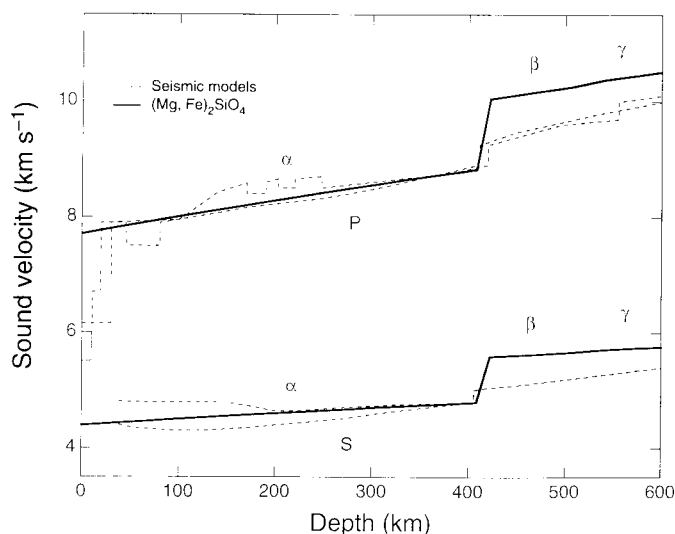


FIG. 3 Sound velocities in $(\text{Mg}_{0.9}\text{Fe}_{0.1})_2\text{SiO}_4$ along a 1,250 °C adiabat compared with seismic velocity profiles^{27–29} for the upper mantle. The solid curve was calculated using $\partial G/\partial T = -0.014 \text{ GPa K}^{-1}$ for the β -phase, consistent with α -phase values. Also included are calculated acoustic velocities in the spinel (γ) phase of $(\text{Mg,Fe})_2\text{SiO}_4$, which is stable below ~ 540 km depth.

profiles. The olivine abundance necessary to satisfy this data is 38%. Allowing the aggregate elastic moduli to vary within the Voigt and Reuss limits changes the calculated divine content by at most $\pm 5\%$.

When the larger value of $\partial G/\partial T$ is used in the calculation, the shear (and to a lesser extent, the compressional) wave velocities in the β -phase are reduced, due to increased temperature sensitivity. The fraction of olivine consistent with seismic data in this case becomes 44% for S waves and 40% for P waves, while remaining at 38% for bulk wave velocity. Thus, even assuming the rigidity of the β -phase is extremely temperature sensitive, current elasticity data for the $(\text{Mg,Fe})_2\text{SiO}_4$ system are not consistent with an olivine-rich composition at 410 km depth.

Our approach assumes that the α - β phase change in a homogeneous mantle composition is the sole cause of the discontinuity. This simplification ignores effects of other possible phase transitions, and changes in element partitioning and the propor-

tions of other phases across the transition. Phase equilibria data¹¹ suggest the last two effects can be neglected to first order. The first effect would only further reduce the amount of olivine required. It is also assumed that there is no texturing of the mineral aggregate, which, if present in the mantle at this depth, could significantly influence our results. The velocity anisotropy of forsterite at 15 GPa remains comparable to ambient-pressure values: 26% for P waves and 21% for S waves. If the pressure derivatives of the β -phase are much lower than present-day measurements suggest, the allowed olivine content of the mantle could be larger. For example, if $\partial K_s/\partial P$ and $\partial G/\partial P$ of the β -phase are assumed to be equivalent to α -phase values (4.2 and 1.4, respectively), the allowed mantle olivine content increases to $\sim 52\%$ (for $\partial G/\partial T = -0.024 \text{ GPa K}^{-1}$). Future studies need to focus on developing a better understanding of the seismic structure of the 410 km discontinuity, as well as characterization of the elasticity of β - Mg_2SiO_4 to higher pressures and temperatures than yet achieved. $\square$

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Preferential predation of female butterflies and the evolution of batesian mimicry

Naota Ohsaki

Entomological Laboratory, Faculty of Agriculture, Kyoto University, Kyoto, 606 Japan

BATESIAN mimicry, in which a palatable mimic resembles an unpalatable model, functions to protect insect mimics from birds. In butterflies that show batesian mimicry, female-limited mimicry is common^{1–3}. The orthodox theory to explain this is sexual selection against males^{4–6}. In these theoretical arguments, no difference in predation pressure between the sexes was assumed, but the existence of female-biased predation would enhance the evolution of sex-limited mimicry. To test for differences in attack rate between the sexes, I examined the rates of beak marks on wings of palatable

butterflies of Papilionidae and Pieridae, and unpalatable Danaidae. Here I report that females were attacked more frequently than males, though danaids were generally attacked less. The papilionid and pierid males had low attack rates similar to those of danaid females. Analysis of a mathematical model highlighted these tendencies. Comparing a batesian mimetic species and its 'model' species, non-mimetic females were selectively attacked and the males, mimetic females and 'models' were attacked less. Therefore females benefit greatly when they become mimetic, whereas males will benefit much less should they become mimetic. Thus female-limited mimicry will be favoured even if the costs of mimicry to both sexes are the same.

In many cases of batesian mimicry, it is the females that are mimetic whereas the males are non-mimetic. There has been no established theory to explain the evolution of female-limited mimicry, although more than 1,500 papers on mimicry have been published this century³. The orthodox theory is that mimicry tends to be limited to females in butterflies as a result of sexual selection against males. However, there is a classic but slighted hypothesis that non-mimetic females are selectively

TABLE 1 Percentage of butterflies with beak marks on wings

	Large damage (%) [*]		Damage including small (%)		Number of captured individuals		
	Males	Females	Males	Females	Males	Females	Females/Males
Papilionidae	28.1	43.4	39.8	54.7	231	53	0.23
Pieridae	23.9	35.9	41.1	55.0	331	120	0.36
Danaidae	14.7	23.2	22.6	35.4	190	99	0.52

Papilionidae, Pieridae and Danaidae include 17, 15 and 17 species, respectively. The genera *Troides* and *Trogonoptera* of Papilionidae and genus *Delias* of Pieridae, most species of which are supposed to be unpalatable, are not included. Therefore, all species of Papilionidae and Pieridae are probably palatable and all species of Danaidae are unpalatable.

^{*} Large marks (≥ 5 mm) were inflicted only by bird attacks in cage experiments, whereas small marks (< 5 mm) were inflicted not only by bird attacks but also by some obstacles such as branches¹².

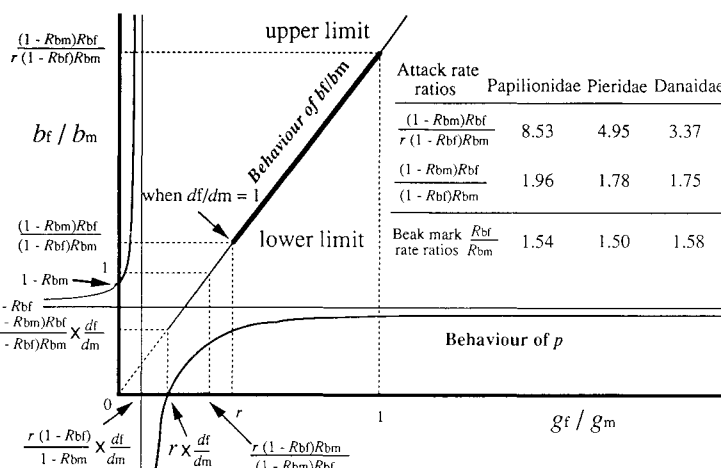


FIG. 1 The behaviour of female/male ratios of estimated attack rates by avian predators (b_f/b_m) as functions of ratios of the collection rates of butterflies (g_f/g_m) and ratios of emigration and mortality except that due to avian predators (d_f/d_m) for the given values of beak mark rates of females (R_{bf}) and males (R_{bm}), and observed sex ratios (r =females/males) of collected butterflies:

$$\frac{b_f}{b_m} = \frac{1}{r} \times \frac{(1-R_{bm})R_{bf}}{(1-R_{bf})R_{bm}} \times \frac{g_f}{g_m} \quad (1)$$

The range of p , which is mortality of butterflies when attacked by avian predators, is also shown:

$$p = (1-R_{bm}) + (R_{bm}-R_{bf}) \left\{ 1 - r \times \frac{1-R_{bf}}{1-R_{bm}} \times \frac{d_f}{d_m} \times \frac{g_m}{g_f} \right\}^{-1} \quad (2)$$

These equations are obtained as follows. Let n_m and n_f be the numbers of males and females in the census area, respectively, a_m and a_f the numbers of new emergences in a unit time, p_m and p_f the mortality when attacked, n_{bm} and n_{bf} the numbers of butterflies with beak marks. d_m , d_f , b_m and b_f are mentioned above. Then the rate equations for the numbers of butterflies in the unit time and those with beak marks are expressed as follows:

$$\frac{dn_m}{dt} = a_m - d_m n_m - p_m b_m n_m$$

$$\frac{dn_{bm}}{dt} = (1-p_m)b_m(n_m - n_{bm}) - d_m n_{bm} - p_m b_m n_{bm}$$

$$\frac{dn_f}{dt} = a_f - d_f n_f - p_f b_f n_f$$

$$\frac{dn_{bf}}{dt} = (1-p_f)b_f(n_f - n_{bf}) - d_f n_{bf} - p_f b_f n_{bf}$$

Suppose the numbers of butterflies in the census period are constant, we obtain in the stationary state:

$$n_m = \frac{a_m}{d_m + p_m b_m}$$

$$n_{bm} = \frac{b_m(1-p_m)}{d_m + b_m} \times n_m = \frac{b_m(1-p_m)}{d_m + b_m} \times \frac{a_m}{d_m + p_m b_m}$$

$$n_f = \frac{a_f}{d_f + p_f b_f}$$

$$n_{bf} = \frac{b_f(1-p_f)}{d_f + b_f} \times n_f = \frac{b_f(1-p_f)}{d_f + b_f} \times \frac{a_f}{d_f + p_f b_f}$$

Therefore, the beak mark rates and the attack rates are given by

$$R_{bm} = \frac{n_{bm}}{n_m} = \frac{(1-p_m)b_m}{b_m + d_m}$$

$$b_m = \frac{R_{bm}d_m}{1-p_m-R_{bm}} \quad (3)$$

$$R_{bf} = \frac{n_{bf}}{n_f} = \frac{(1-p_f)b_f}{b_f + d_f}$$

$$b_f = \frac{R_{bf}d_f}{1-p_f-R_{bf}}$$

Let g_m and g_f be so that the numbers of butterflies collected are given by

$$N_m = g_m n_m = \frac{g_m a_m}{d_m + p_m b_m}$$

$$N_f = g_f n_f = \frac{g_f a_f}{d_f + p_f b_f}$$

Therefore, the observed sex ratio is given by

$$r = \frac{N_f}{N_m} = \frac{g_f n_f}{g_m n_m} = \frac{g_f a_f (p_m b_m + d_m)}{g_m a_m (p_f b_f + d_f)}$$

If the numbers of emerging females and males in the unit time are equal, $a_m = a_f = a$, and if the mortality of females and males attacked are equal, $p_m = p_f = p$, the above equation becomes

$$r = \frac{g_f(p b_m + d_m)}{g_m(p b_f + d_f)} \quad (4)$$

This equation indicates that there are three factors that cause male-biased observed sex ratios ($r < 1$): mortality by avian predation ($p b_f / p b_m > 1$), emigration and mortality except that due to avian predation ($d_f / d_m > 1$), and collection rates ($g_f / g_m < 1$). From equations (3) and (4), we obtain the expression for b_f/b_m and p in equations (1) and (2).

Because $p \geq 0$, b_f/b_m has the minimum of

$$\frac{(1-R_{bm})R_{bf}}{(1-R_{bf})R_{bm}} \times \frac{d_f}{d_m}$$

which is less than one. This value is reached when $g_f/g_m = r \times d_f/d_m$. Therefore when d_f/d_m is extremely low, b_f/b_m may conceivably become less than one, and males are more frequently attacked by avian predators than females, even though beak mark rates of females are higher than males.

However, field studies on the causes of male-biased observed sex ratios of butterflies estimate that d_f/d_m is greater than 1. In particular the emigration rate of females has been estimated to be much higher than males^{10,11}. Therefore, I assume that $d_f/d_m = 1$ is its lower limit. Then, the lower limit of b_f/b_m is estimated to be

$$\frac{(1-R_{bm})R_{bf}}{(1-R_{bf})R_{bm}}$$

when $g_f/g_m = r$.

The upper limit of b_f/b_m is governed by the upper limit of g_f/g_m , but there are few field studies that estimate g_f/g_m . However, $g_f/g_m < 1$ is one of the factors that causes male-biased observed sex ratios. Therefore, the value of b_f/b_m when $g_f/g_m = 1$ may be assumed as the standard of upper limit of b_f/b_m :

$$\frac{1}{r} \times \frac{(1-R_{bm})R_{bf}}{(1-R_{bf})R_{bm}}$$

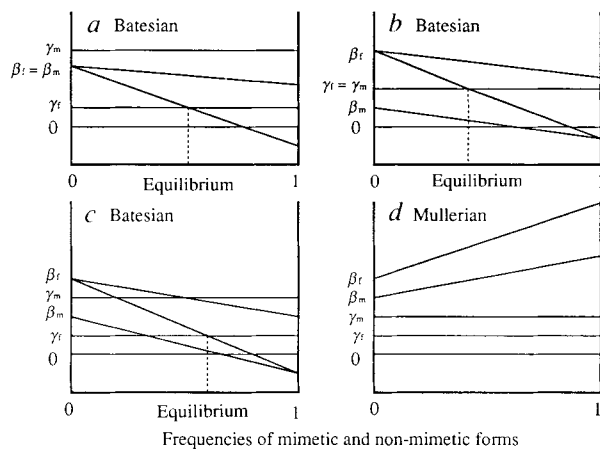


FIG. 2 Frequency of mimetic and non-mimetic forms in a batesian (palatable) species (a–c) and a mullerian (unpalatable) species (d). β_r and β_m are the benefits for mimicry to females and males, respectively, and γ_r and γ_m are the costs. When $\beta_r - \gamma_r > 0$ and/or $\beta_m - \gamma_m > 0$, females and/or males may become mimetic. When $\beta_r - \gamma_r < 0$ and/or $\beta_m - \gamma_m < 0$, females and/or males remain non-mimetic. In batesian mimicry, the benefits of a mimetic form is dependent on its numbers, not on its frequency, but the benefits decrease when the frequency of mimics increase in the population. When there are many models and population size of the mimetic species is small, the frequency of mimetic forms increase. In mullerian mimicry, the benefits are positively density-dependent and increase when the frequency of mimics increase.

attacked by avian predators, but males are rarely attacked^{2,7}, and therefore that female-limited mimicry occurs even when the costs of mimicry are the same for each sex. Attempting to examine the hypothesis, I collected 17 Papilionidae and 15 Pieridae which are considered to be palatable, and 17 Danaidae which are considered to be unpalatable⁸, in Borneo, Malaysia.

The rate of beak marks, that is, wing damage indicative of predator attacks, in females was higher than in males in all three families (χ^2 test; d.f. = 1, $P < 0.05$ except Danaidae), which suggests that females are selectively attacked. However, the beak mark rates of danaids were lower than those of the other families in each sex (χ^2 test; d.f. = 1, $P < 0.05$ in each case), suggesting that avian predators recognized their unpalatability and avoided them. The beak mark rates of papilionid and pierid males were also low and similar to those of danaid females (χ^2 test; d.f. = 1, $P > 0.05$ in each case). Sex ratios of the collected butterflies were male-biased in the three families (Table 1).

Because beak marks are indicative of predation attacks and failure, it is very difficult to interpret the meaning of beak mark frequencies in butterflies⁹. I estimated the lower and upper limits of the actual attack rate ratios using a simple mathematical model from the beak mark rates and sex ratios of collected butterflies (Fig. 1). Presumably, actual attack rate ratios are between the two estimated values. These two values calculated for the three families show that actual attack rate ratios are more biased to females than beak mark rate ratios (Fig. 1), suggesting that females are selectively attacked. Females are often heavier and less agile in flight than males because they carry eggs, also females often hover around oviposition plants, and may be more liable to be caught by birds.

I compared the beak mark rates of *Papilio polytes* Linné, a batesian mimetic species, and *Pachliopta aristolochiae* Fabricius,

TABLE 2 Percentage of butterflies with beak marks on wings of batesian mimetic species and the model one

Species	Types	Number of individuals		Beak mark (%)
		Beak mark	No mark	
<i>P. polytes</i>	Males	29	97	23
	Non-mimetic females	8	7	53
	Mimetic females	7	18	28
	Models	2	5	29
<i>P. aristolochiae</i>	Males	1	5	
	Females	1	0	

Only large marks (≥ 5 mm) are recognized as beak marks. Large marks (≥ 5 mm) were inflicted only by bird attacks in cage experiments, while small marks (< 5 mm) were inflicted not only by bird attacks but also by some obstacles such as branches¹².

on which it models itself (Table 2). The beak mark rate of non-mimetic females of *P. polytes* was high (Fisher's exact probability test; $P < 0.05$ versus males, $P > 0.05$ versus mimetic females and models), whereas that of its mimetic females was low, similar to that of the models (Fisher's exact probability test; $P > 0.05$). In addition, the beak mark rate of male *P. polytes* was as low as those of mimetic females and models (χ^2 test; d.f. = 1, $P > 0.05$ in each case). If males had been mimetic, their beak mark rates would probably have been as low as those of danaid males (Table 1). Therefore, considering their actual attack rates, females benefit greatly when they become mimetic, whereas males will benefit much less even if they become mimetic. One reason why males do not become mimics could be that mimicry is costly and that the costs to males may outweigh the benefits, so that male-specific modifiers that prevent mimicry will evolve. There is no obvious reason why there should be a cost, and the cost to males is usually considered to be sexual selection acting on males against changed colours.

In batesian mimicry, the benefits of a mimetic form are density-dependent, not frequency-dependent. However, for a given population size the benefit to mimetic females decreases when the frequency of mimetic females increases in the population. When the benefits and costs are in equilibrium, the frequencies of mimetic and non-mimetic females are also in equilibrium. When there are many models and the population size of the mimic species is small, the frequency of mimetic females increases and all females eventually become mimetic (Fig. 2a). The beak mark rate of non-mimetic females of *P. polytes* was higher than that of mimetic ones (Table 2), which may be due to either or both of two causes. One is that the frequency of mimetic females in the population was smaller than the equilibrium frequency. Another is that the frequency of mimetic females was in equilibrium, and that even the cost to mimetic females is very large.

Thus, when the predation by avian predators is female-biased, female-limited mimicry will evolve even if the costs to both sexes are the same (Fig. 2b). Of course, when there is more sexual selection against males, the difference between benefits and costs in females is larger than in males (Fig. 2c). In mullerian mimicry, in which unpalatable species resemble each other, all individuals in both sexes usually become mimetic^{1,3}, as the density-dependent benefits to mimics increase with the increasing frequency of mimicry for a given population size (Fig. 2d). □

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A functional neuroanatomy of hallucinations in schizophrenia

D. A. Silbersweig^{*†‡¶}, **E. Stern**^{*†‡}, **C. Frith**^{*†},
C. Cahill^{*}, **A. Holmes**[‡], **Sylke Grooten**^{*†},
J. Seaward^{*}, **P. McKenna**[§], **S. E. Chua**^{*},
L. Schnorr^{*}, **T. Jones**^{*} & **R. S. J. Frackowiak**^{*†‡}

^{*} MRC Cyclotron Unit, [‡] Wellcome Department of Cognitive Neurology, Institute of Neurology, Hammersmith Hospital, Duane Road, London W12 0NN, UK

[†] Functional Neuroimaging Laboratory, Box 171, The New York Hospital-Cornell Medical Center, 525 East 68th Street, New York, New York 10021, USA

[§] Fulbourn Hospital, Cambridge Road, Fulbourn, CB1 5EF, UK

HALLUCINATIONS, perceptions in the absence of external stimuli, are prominent among the core symptoms of schizophrenia. The neural correlates of these brief, involuntary experiences are not well understood, and have not been imaged selectively. We have used new positron emission tomography (PET) methods^{1,2} to study the brain state associated with the occurrence of hallucinations in six schizophrenic patients. Here we present a group study of five patients with classic auditory verbal hallucinations despite medication, demonstrating activations in subcortical nuclei (thalamic, striatal), limbic structures (especially hippocampus), and paralimbic regions (parahippocampal and cingulate gyri, as well as orbito-frontal cortex). We also present a case study of a unique, drug-naïve patient with visual as well as auditory verbal hallucinations, demonstrating activations in visual and auditory/linguistic association cortices as part of a distributed cortical-subcortical network. Activity in deep brain structures, identified with group analysis, may generate or modulate hallucinations, and the particular neocortical regions entrained in individual patients may affect their specific perceptual content. The interaction of these distributed neural systems provides a biological basis for the bizarre reports of schizophrenic patients.

We have studied hallucinations in a group of five right-handed patients (mean age 31.8, range 25–42 years) with a DSM IV (DSM, diagnostic and statistical manual of mental disorders) diagnosis of schizophrenia (paranoid type, continuous), using new PET methods developed and validated for the imaging of such transient, subjective, randomly occurring neuropsychiatric symptoms^{1,2}. The patients were all afflicted with frequent, classic auditory verbal hallucinations (of voices talking to or about them) despite receiving neuroleptic medication. Hallucinations were also studied in a clinically distinct patient who was never medicated and was experiencing frequent hallucinations in both the visual and auditory modalities. This patient was a 23-year-old right-handed male with a DSM IV diagnosis of schizophrenia (paranoid type) of 2 years duration. He saw moving, coloured scenes with rolling, disembodied heads, and simultaneously heard the heads speaking to him, giving him instructions.

Each patient's regional cerebral blood flow (rCBF) distribution was recorded as an index of local neuronal activity using a low-dose, slow-bolus H₂¹⁵O PET technique¹. For each of 22–25 scans performed in two study sessions (10–13 scans per session), the patients were instructed to relax, close their eyes, and press a button during the voices (which were usually accompanied by visions in the drug-naïve patient). An event-related count-rate correlational analysis² was performed on the images within the framework of pixel-by-pixel statistical parametric mapping (SPM)³ (see Fig. 2 legend). Brain areas in which activity was significantly associated with the hallucinatory events were thereby identified (Figs 1 and 2).

TABLE 1 Local statistical maxima in the pattern of brain activity during auditory verbal hallucinations in the group study

Area (Brodmann's area)	x, y, z* (mm)	Z score
Right thalamus	14, -10, 4	5.76†
Right putamen	18, 4, -4	5.68†
Left parahippocampal gyrus (27, 30)	-14, -38, 0	4.91†
Left thalamus	-12, -20, 8	4.63†
Right caudate (body)	12, -2, 20	3.84
Left cerebellum (vermis)	-6, -46, -8	3.79
Right anterior cingulate (24)	12, 10, 32	3.78
Left subcallosal gyrus (25)	-8, 28, -16	3.60
Right parahippocampal gyrus (36)	26, -44, -8	3.10

These areas are above significance thresholds of $P=0.001$, by reference to the unit normal distribution ($Z=3.09$), and most are above $P=0.0001$ ($Z=3.72$). The areas of activation with maxima in the parahippocampal gyrus include the adjacent hippocampus. The area of activation with a maximum in the right ventral putamen includes the adjacent nucleus accumbens.

* The coordinates are relative to the anterior commissure in the stereotactic space defined by Talairach and Tournoux; x is the lateral distance from the midline (positive, right), y is the anteroposterior distance from the anterior commissure (positive, anterior), and z is the height relative to the intercommissural line.

† $P<0.005$, corrected.

In the group of five patients with auditory verbal hallucinations, maximal activity was localized to the bilateral thalamus, left hippocampus/parahippocampal gyrus, and right ventral striatum. Right hippocampus/parahippocampal gyrus, right anterior cingulate, and left orbitofrontal cortex were also included in a pattern of significant activation (Table 1).

In the patient with hallucinations in two sensory modalities, the pattern of significantly increased brain activity included areas in bilateral visual, auditory and multimodal association cortices (left more extensive than right, corresponding to Brodmann's areas (BA) 18, 19, 37, 21, 22 and 39); paralimbic cortices (left posterior cingulate gyrus corresponding to BA 31, and right parahippocampal gyrus and temporal pole); right precuneus, putamen and superior colliculus; left primary sensorimotor cortices (in regions corresponding to the representation of the right thumb (button press)) and premotor cortex; and bilateral anterior cerebellum (Table 2).

These results demonstrate the complementary nature of group and individual analyses, and suggest an explanation as to how a schizophrenic patient's experiences may be determined by aberrant brain activity. In the group analysis a highly significant common pattern of deep activations emerged. These deep structures are highly interconnected, and have demonstrated cytoarchitectonic and morphometric abnormalities in studies of schizophrenia^{4–6}. They may play a central role in the generation of, modulation of, or response to, neocortical activity during hallucinations. Although the thalamus is often considered to be the gate to the forebrain, it has been suggested that the majority of thalamocortical connections are devoted to generating an internal representation of reality, in the presence or absence of sensory input⁷. The parahippocampal gyrus and adjacent hippocampus and cingulate gyrus have widespread connections with limbic regions and association cortices, providing a meeting point for internal and affective data with current and past sensory representations⁸. Increased dopaminergic transmission in the ventral striatum⁹ and glutamatergic dysfunction¹⁰ are thought to be involved in the disinhibition of these cortical-subcortical circuits, leading to pathological releases of behaviour and experience during psychotic episodes in schizophrenia. Increased rCBF in all of these deep regions has been correlated (especially on the left) with psychopathology in schizophrenia¹¹.

Neocortical activations were absent from the group result, but present (including temporoparietal auditory-linguistic association cortex) in each individual subject (manuscript in prepara-

¶ To whom correspondence should be addressed in New York.

tion). There was inter-subject variability in the precise location of these neocortical activations, perhaps reflecting differences in the exact sensory content and experience of hallucinations from patient to patient, and resulting in a lack of constructive interference in the group result. Conversely, activation of subcortical and (para)limbic structures may be less variable (both spatially and functionally) and more readily detected with the increased power of group analyses.

The case study allowed the identification of activations highly specific to the perceptual content of hallucinations in an indi-

vidual patient. Visual and auditory verbal hallucinations were associated with activity in visual association cortex (specialized for higher-order visual perception) and auditory-linguistic association cortex (specialized for speech perception). Visual^{1,2} and auditory-linguistic² activation studies of normal subjects have demonstrated activations of similar sensory association cortices, as well as primary cortices. Localization in association rather than primary cortex in this patient is consistent with the complex and internally generated nature of his perceptual experiences. This finding also substantiates the position that activity in pri-

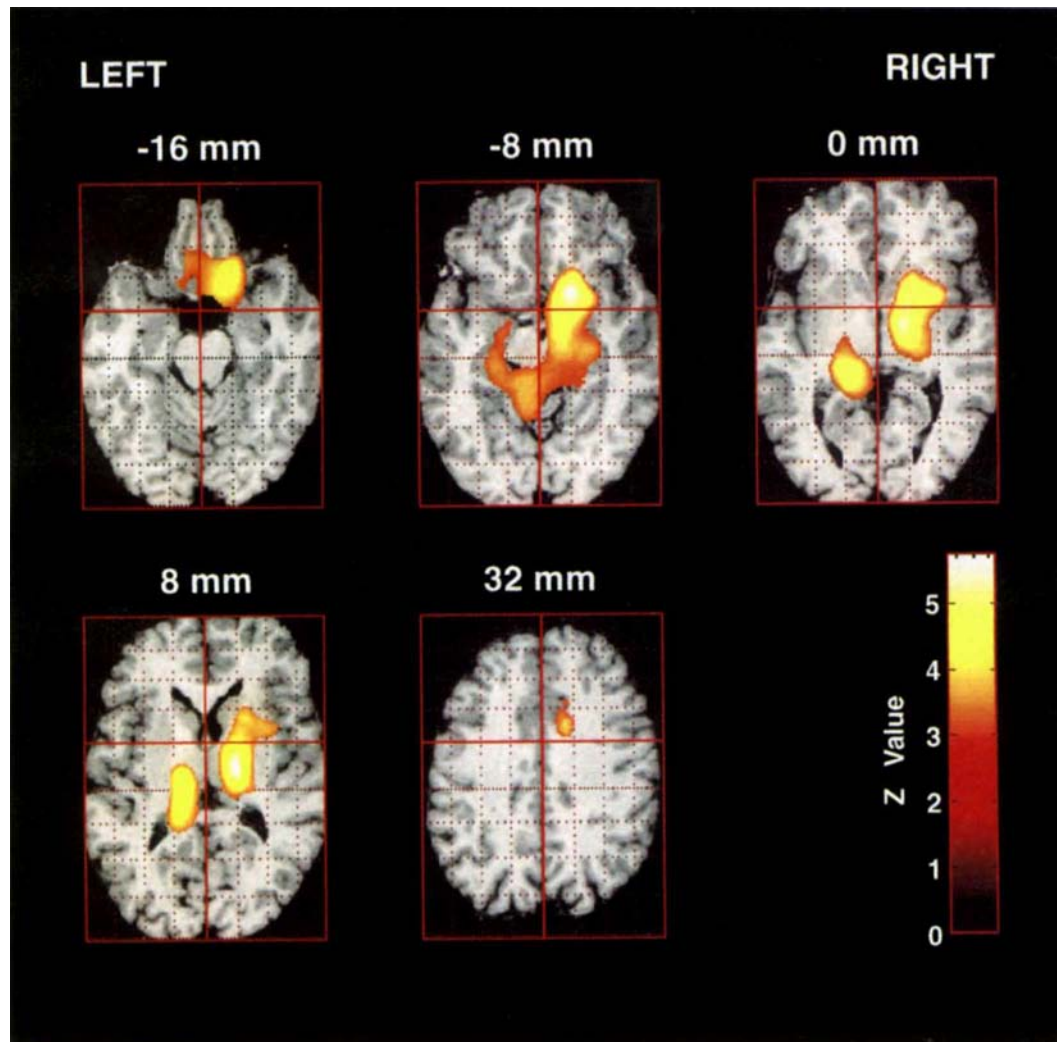


FIG. 1 Axial sections demonstrating brain areas with significantly increased activity during auditory verbal hallucinations in the group study. Functional PET results (threshold at $Z = 3.09$, or $P < 0.001$, by reference to the unit normal distribution) are displayed in colour, superimposed upon a single structural T1-weighted magnetic resonance imaging (MRI) scan that has been transformed into the Talairach space for anatomical reference. Section numbers refer to the distance from the anterior commissure–posterior commissure line, with positive numbers being superior to the line. The areas of maximal activation are described in the text and listed in Table 1. The areas of activation extend into the amygdala bilaterally, and into the right orbitofrontal cortex. Although these regions of extension are consistent with the limbic/paralimbic component of activity during hallucinations, and may contribute to drive and affect in this context, definitive statements cannot be made in the absence of discrete maxima.

METHODS. The within-subject component of the methods is the same as that described in the Fig. 2 legend, except for a transformation of these images into the stereotactic space of Talairach and Tournoux²⁹, and the use of a $15 \times 15 \times 15$ mm gaussian filter. The group analysis was performed by determining the significance of the covariate (halluci-

nation scan score) effect, or overall correlation, at each voxel, in a linear model including an effect for global CBF, and additive subject effects; the latter adjust for between-subject differences in mean regional CBF not accounted for by global changes. Note that the patients in the group were medicated with neuroleptics, and some were also on other low-dose medications for side effects or occasional mild sedation (the latter not at the time of scanning). Although a medication effect should always be considered, it does not influence the analysis, which is based upon the within-subject variance induced by the target events of hallucinatory activity in the setting of (and despite) constant levels of medication. The group component of the analysis is also based on the hallucination covariate of interest and is normalized for subject effects on mean regional CBF. The possible neural correlates of the button-press report should also be considered. The left primary motor-sensory cortex (seen in the individual coregistered images of Fig. 2) is clearly involved in this process. The cerebellum may be involved in higher as well as motor processes. Motor correlates are also possible in subcortical nuclei, but the right ventral location of the striatal activation and the bilateral nature (and posterior left location of) thalamic activation suggest that these regions are primarily associated with the hallucinatory activity.

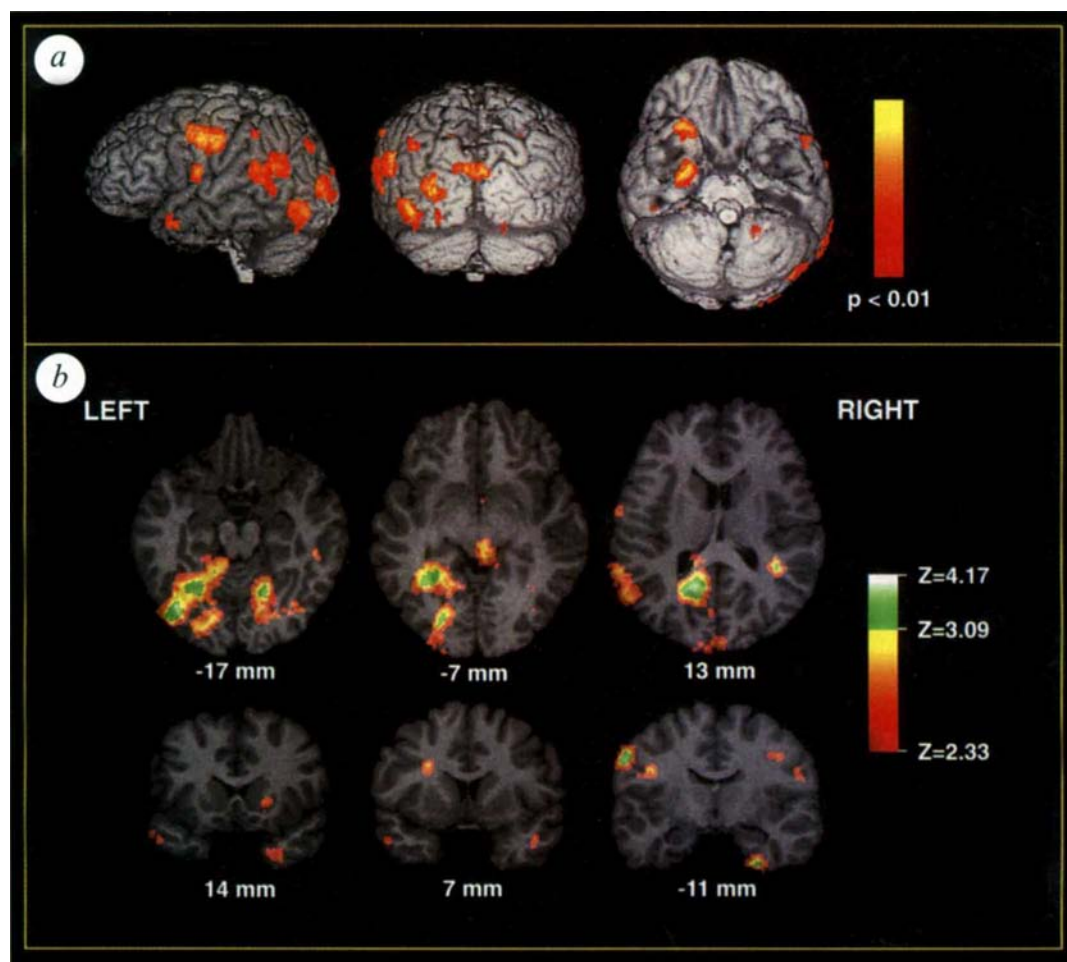


FIG. 2 Surface projections (a; left lateral, posterior and ventral) and sections (b; top row axial, bottom row coronal) of brain areas with significantly increased activity during visual and auditory verbal hallucinations in the case study. Functional PET results (thresholded at $P < 0.01$ ($Z = 2.33$) and $P < 0.001$ ($Z = 3.09$), by reference to the unit normal distribution) are shown in colour, superimposed upon the structural T1-weighted MRI scan. The selected sections illustrate several activations and not necessarily the centre of each activation. Section numbers refer to the distance from the anterior commissure, with positive numbers being superior to the anterior commissure for the axial sections, and anterior to it for the coronal sections. The areas of activation are described in the text and listed in Table 2.

METHODS. rCBF was measured (as an index of neuronal activity) with a Siemens/CPS 953B PET scanner in high-sensitivity three-dimensional mode using a low-dose $H_2^{15}O$ slow-bolus technique¹. The data were corrected for background activity and attenuation, reconstructed (Hanning filter 0.5, 8.4-mm resolution FWHM), and the images were realigned to one another, trimmed to remove scalp artefact, smoothed with a $15 \times 15 \times 9$ mm gaussian filter, and normalized using analysis of covariance to remove the effect of differences in global blood flow across scans or sessions. The patient was studied during two sessions

within one month. He was carefully selected (as were all of the patients) for his frequent target symptoms, his cooperative and reliable nature, and his consistent performance in prescan practice of self-reporting. For each of 25 scans (13 in the first session, 12 in the second), he was asked to relax, close his eyes and press a button with his right thumb during each hallucination. The timing and duration of each hallucination in relation to the 30 s of radiotracer delivery (the exact timing of which was unknown to the patient) was logged by computer. This information was used to derive a weighted score for each scan, reflecting the contribution of radiotracer deposition during hallucinations to the image². There was a spread of scan scores over the study sessions, reflecting differences across scans in the exact timing, duration and frequency of hallucinations, as well as the dynamic nature of the radiotracer input function during each scan. An event-related count-rate correlational analysis² was then performed within the framework of pixel-by-pixel SPM³. Without the need for 'non-hallucinating control scans', this analysis identifies pixels with intensities covarying with the scores, corresponding to areas of the brain in which activity is specifically associated with the hallucinations. The functional PET findings were coregistered³⁰ with a T1-weighted structural MRI scan of the patient's brain for accurate neuroanatomical localization.

mary cortices is not a necessary prerequisite for conscious perception¹³. The dominant hemisphere cortical activations may reflect the characteristic linguistic content of hallucinations in schizophrenia. Left-hemisphere predominance has also been noted in some studies of mental imagery¹⁴, a perceptual state which (like hallucinations) is self-generated, but which (unlike hallucinations) is voluntary. Posterior cingulate cortex, prominently activated in the patient with visual hallucinations, has (with the precuneus) been implicated in visual imagery and episodic memory recall¹⁵, and is known to be affected by psychotomimetic drugs¹⁰. Imagery however, is associated with a pattern

of neural activity different from that described here, involving premotor cortex as well as post-rolandic regions in an auditory verbal imagery experiment¹⁶.

The relative absence of activation in prefrontal regions in the presence of striking post-rolandic activity may be an important dissociation in this patient, reflecting decreased functional connectivity and resulting in a lack of inhibition, volitional control or internal monitoring¹⁷. This is consistent with reported hypo-frontality in schizophrenia¹⁸, and may be involved in the patient's misattribution of involuntary, internally generated percepts. The involvement of non-dominant paralimbic/subcortical systems

TABLE 2 Local statistical maxima in the pattern of brain activity during visual and auditory verbal hallucinations in the case study

Brain region (Brodmann's area)	Left		Right	
	x, y, z coordinates (mm) (relative to anterior commissure)	Z score	x, y, z coordinates (mm) (relative to anterior commissure)	Z score
Association cortices				
lingual gyrus (18, 19)			18, -73, -24	4.14
fusiform gyrus (19, 37)	-41, -62, -23	3.99	32, -54, 0	2.97
	-43, -72, -18	3.96	47, -63, -25	2.80
	-30, -60, -12	3.54		
	-26, -85, -16	2.79		
inferior occipital gyrus (18, 19)	-27, -82, -6	3.89	43, -75, -17	2.43
middle occipital gyrus (18)	-35, -98, -1	3.38		
posterior superior temporal gyrus (22)	-70, -47, 13	2.76	41, -44, 16	3.52
anterior middle temporal gyrus (21)	-54, 14, -25	2.44	44, 7, -28	2.55
posterior inferior temporal gyrus (20)			52, -37, -17	2.48
angular gyrus (39)	-60, -64, 14	2.95		
precuneus (7)			11, -59, 30	2.78
precentral gyrus (6)	-26, 3, 26	2.93	56, -10, 22	3.03
	-70, -6, 10	2.97		
medial middle frontal gyrus (8)	-2, 35, 39	2.58		
Paralimbic cortices				
posterior cingulate gyrus (31)	-14, -63, 15	4.17	29, -12, -38	3.43
parahippocampal gyrus (28)			28, 18, -37	2.61
anterior superior temporal gyrus (38)				
Primary motor cortex				
precentral gyrus (4)	-64, -14, 33	4.06	45, -5, 36	3.29
Others				
cerebellum	-13, -45, -16	3.43	33, -51, -31	2.83
	-17, -47, -38	3.39		
anterior putamen			21, 13, 1	2.75
superior colliculus			3, -38, -5	3.23

Table entries indicate the location and significance of local statistical maxima in the pattern of brain activation associated with hallucinations and the button-press report. The coordinates are relative to this patient's anterior commissure (they are not in Talairach space); x is the lateral distance from the midline (positive, right), y is the anteroposterior distance from the anterior commissure (positive, anterior), and z is the height relative to the intercommissural line. All areas are included in an SPM thresholded at a level of $P < 0.01$, by reference to the unit normal distribution. Z scores above 3.09 are significant at $P < 0.001$.

which subsume attentional and motivational functions¹⁹ may also contribute to the sense of reality and the emotional relevance ascribed to the experiences.

The findings presented here extend those of previous PET^{20, 23} and single photon emission computed tomography (SPECT)²⁴⁻²⁸ studies of schizophrenic patients with hallucinations. Previous studies utilized lower-sensitivity image acquisition and region-of-interest analysis, examining 'resting' brain activity in patients with a history of hallucinations, or brain activity in patients with recent reports of hallucinatory activity. A more specific image of the hallucinating brain could not be obtained because the methods used did not allow the isolation of the transient, involuntary target symptom from concurrent symptoms or mental states. The results of these earlier studies are varied. When considered together, however, they implicate several of the regions identified in our experiments, including auditory-language cortices, and bilateral limbic and paralimbic regions. The particular, symptom-specific patterns of neural activity noted above, the prominent thalamic involvement (in the group), and the lack of activation in Broca's area are among the distinguishing features of the current studies. The latter finding suggests that, although inner speech may be involved, perhaps it is not the central pathological process in symptom formation.

In conclusion, the group and single-subject studies reported here highlight different aspects of brain states specifically during hallucinations in schizophrenia. The group findings demonstrate a common pattern of deep brain activity across five patients with classic auditory verbal hallucinations. The individual findings in a clinically distinct patient provide an example of specific neocortical activations consistent with the visual and auditory verbal content of his hallucinations. Autonomous activity in these distributed neural systems is consistent with a growing body of scientific literature on schizophrenia, and can account for the bizarre, involuntary experiences of these patients. These

findings may also contribute, at the systems level, to our understanding of the neural substrate of conscious perceptual and emotional experience. □

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Dopaminergic modulation of impaired cognitive activation in the anterior cingulate cortex in schizophrenia

R. J. Dolan^{*†}, P. Fletcher^{*†}, C. D. Frith^{*†},
K. J. Friston^{*†}, R. S. J. Frackowiak^{*}
& P. M. Grasby[§]

^{*}Wellcome Department of Cognitive Neurology, Institute of Neurology, Queen Square, London WC1N 3BG, UK

[†]Royal Free Hospital School of Medicine, Roland Hill Street, London NW3, UK

[‡]Department of Psychology, University College London, Gower Street, London WC1E 6BT, UK

[§]MRC Cyclotron Unit, Hammersmith Hospital, Ducane Road, London W12 0HS, UK

DOPAMINERGIC dysregulation remains an empirical cornerstone for theories concerning the causation of schizophrenia. Evidence for a dopamine system dysfunction in schizophrenia includes the psychosis-inducing effects of dopaminergic agonists^{1,2} and the antipsychotic potency of dopaminergic antagonists^{3,4}. Here we use positron emission tomography (PET) to examine the regulatory role of dopamine on cortical function in normal subjects and unmedicated schizophrenic patients. Using a factorial experimental design, we compared the effect of dopaminergic manipulation with apomorphine on a neural response to a cognitive task. In the schizophrenic patients, relative to controls, an impaired cognitive activation of the anterior cingulate cortex was significantly modulated by a manipulation of dopaminergic neurotransmission. Thus, after apomorphine, the schizophrenic subjects displayed a significantly enhanced cognitive activation of the anterior cingulate cortex relative to the controls. These data provide *in vivo* evidence that an impaired cognitive-task-induced activation of the anterior cingulate cortex in schizophrenic patients can be significantly modulated by a dopaminergic manipulation.

We measured brain activity, indexed by regional cerebral blood flow (rCBF), in 12 normal male volunteer subjects and 12 unmedicated male schizophrenic patients under two conditions. We scanned all subjects while they performed a paced verbal fluency task and a baseline paced verbal repetition task using an established protocol⁵. Under the same experimental conditions, we scanned the subjects before (scans 1 and 2) and after (scans 3–6) an injection of either the non-selective dopaminergic agonist apomorphine or a placebo. Six separate rCBF measurements, with an interscan interval of 10 minutes, were thus recorded from each subject. The overall experimental design is summarized in Fig. 1. This factorial design enabled us to determine from the interaction term the effect of one treatment (the administration of the dopaminergic agonist apomorphine) on the effect of the other (the neuronal response to a cognitive activation). In this particular experiment, the effect of interest is the differential effect across groups of dopamine perturbation on the pattern of neuronal activation induced by the cognitive activation.

The cognitive activation defined the neural system to be modulated by apomorphine administration. In normal subjects the cognitive activation, derived from the subtraction of the neuronal response in the repetition condition from the fluency condition, showed the expected pattern of activation that included the left dorsolateral prefrontal cortex, the thalamus and the anterior cingulate cortex⁵. The same cognitive activation in schizophrenic patients indicated a failure of activation that was localized to the anterior cingulate cortex ($P < 0.05$, corrected for multiple comparisons) (Fig. 2a). After dopaminergic manipulation with apomorphine, this relative failure of task-induced cingulate acti-

vation in schizophrenic patients was reversed. In the post-apomorphine state, schizophrenic patients displayed a significant augmentation of the neuronal response to cognitive activation in the anterior cingulate cortex relative to control subjects ($P < 0.0001$, uncorrected) (Fig. 2b). This effect was specific for verbal fluency-induced activation as no differential effect of apomorphine on patterns of activation was evident under the repetition condition alone ($P > 0.05$ uncorrected). The changes in rCBF at a selected pixel, pre- and post-apomorphine, during cognitive activation for controls and schizophrenic patients are shown in Fig. 3. A restriction of the analysis to the nine medication-naïve subjects provided findings identical to those of the whole group.

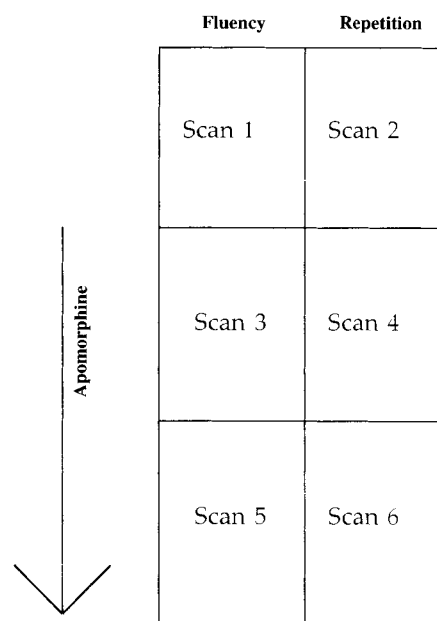


FIG. 1 Experimental design. Each square refers to a single measurement of rCBF. The cognitive task consisted of a paced orthographic verbal fluency (scans 1, 3 and 5) or a verbal word-repetition control task (scans 2, 4 and 6). The tasks required a verbal response every 5 s, so equality of response rate was ensured across trials and groups. The cognitive activation, defined by the subtraction of the repetition control from the fluency conditions, activates a prefrontal network that includes the dorsolateral prefrontal cortex and the anterior cingulate cortex⁵. The drug manipulation consisted of apomorphine, given subcutaneously in a dosage of $10 \mu\text{g kg}^{-1}$, or placebo administered before scan 3. This dosage has a significant effect on neural activity in the prefrontal and anterior cingulate cortex²⁵. Control subjects and schizophrenic patients were therefore studied under a condition of verbal fluency and verbal repetition both before and after a subcutaneous injection of either apomorphine or placebo. Thus 6 of the 12 controls and 6 of the 12 schizophrenics received placebo, and the remaining 6 controls and 6 schizophrenics received apomorphine, before scan 3. The cognitive activation within a group is specified by the contrast of scans $(1 - 2) + (3 - 4) + (5 - 6)$. The drug-induced augmentation of the cognitive activation, within a group, is specified by the contrast $[(3 - 4) + (5 - 6)]/2 - (1 - 2)$. A between-group comparison is the difference in the magnitude of this effect across groups. We studied 24 subjects, all of whom were right-handed as determined by the Edinburgh Handedness Inventory²⁶. These comprised 12 unmedicated patients who met DSM III-R criteria for schizophrenia. The mean age of the patients was 26 years (s.d., 7), and the mean duration of illness was 4.3 years (s.d. 6). Nine of the patients were neuroleptic and psychotropic-medication-naïve, while the remaining three were free of psychotropic medication for a minimum of six months. The 12 control subjects, with a mean age of 25 years (s.d., 4), were volunteers recruited through advertisement. None of the controls had any past psychiatric or medical illness.

These observations can be considered in the context of the neuromodulatory functions of dopamine, which has a critical regulatory role on prefrontal neurophysiology^{6,7}. A disorder of this regulatory function is thought to account for a range of human pathologies including the psychoses⁸. The effects of dopamine on cortical function are complex, although the net effect is inhibitory⁹. However, its critical effects are neuromodulatory, as exemplified by observations that the electrophysiological responses of populations of task-activated prefrontal neurons are further enhanced by dopamine¹⁰. We found a significant neuromodulatory effect of dopamine manipulation on the neuronal response to cognitive activation in the anterior cingulate cortex of unmedicated schizophrenic patients. A significant effect of apomorphine on glucose metabolism in the striatum in drug-naïve schizophrenics under non-activation conditions has previously been reported¹¹. We found a modulatory effect of dopamine which was activation-task specific, and was not seen under the repetition condition. These findings suggest that dopaminergic inputs, either direct or indirect, have a differential regulatory effect on anterior cingulate activity in schizophrenics compared to controls.

One model of dopamine dysfunction in schizophrenia has suggested diminished tonic dopamine release, upregulation of postsynaptic dopamine receptors, and a resulting increase in postsynaptic responsiveness to phasic dopamine activation¹². As apomorphine at the dose administered has significant postsynaptic actions, the observed impairment of pre-drug task activation and the augmented post-drug task activation are consistent with this model. The apparent regional specificity of our finding is also in accord with evidence from functional imaging¹³⁻¹⁵ and other investigative modalities which indicate cingulate pathology in schizophrenia^{16,17}.

The anterior cingulate cortex has multiple functional specializations, including a role in attentional mechanisms, which are core abnormalities in schizophrenia^{15,18,19}. The predominant dopamine inputs to the cingulate are to layer II and III pyramidal cells, which are the primary cortico-cortical output layers²⁰. A disorder of cortico-cortical integration in schizophrenia has been suggested by PET findings of abnormal functional connectivity between the frontal and temporal cortices^{21,22}. The anterior cingulate may participate, directly or indirectly, in cortico-cortical integration. A direct modulatory effect of cingulate

FIG. 2 a, Brain regions showing a significantly greater neuronal response to a cognitive activation (verbal fluency and repetition) in normal subjects compared to schizophrenic subjects in the placebo groups alone. We present a statistical parametric map (SPM) thresholded at $P < 0.001$ (uncorrected). The region showing a significant difference exceeds a P -value that has been corrected for the volume analysed using standard results based upon the theory of gaussian fields²⁷. The data show significantly greater activation of the anterior cingulate cortex in control subjects ($P < 0.05$, corrected). b, Comparison of brain regions with a significantly greater neuronal response to the same cognitive activation in schizophrenic subjects compared to normals following injection of apomorphine. The SPM reflects the apomorphine-dependent effects and shows only voxels that were significant using two orthogonal or independent contrasts. The first contrast reflects the cognitive activation (in the scans without apomorphine) and the second tests for an apomorphine enhancement (for voxels that were significant in the first contrast) of the cognitive activation that was greater in the schizophrenics than normals. An uncorrected value of 0.01 was used for both these contrasts. Because of this independence, the probability of the resulting voxels surviving these joint criteria by chance is 0.0001 (uncorrected). The data show that during cognitive activation patients with schizophrenia have an enhanced neuronal response in the anterior cingulate cortex after apomorphine injection. The coordinates of the site of maximal interaction are $x = +8$, $y = +18$, $z = +28$. The images are SPMs superimposed onto a normal magnetic resonance image (MRI) which has been normalized into a standard stereotactic space²⁸. Views of the brain are shown for orthogonal slices (transverse, coronal and sagittal) at the pixel of maximal interaction. The study was approved by the local hospital ethics committee and ARSAC(UK). Scans were obtained using a CTI model 953B PET Scanner (CTI, Knoxville, USA) with collimating septa retracted (FWHM 8 mm transaxial, 4 mm axial). Volunteers received a 20 s intravenous bolus of $H_2^{15}O$ at a concentration of 55 MBq ml⁻¹ and a flow rate of 10 ml min⁻¹ through a forearm cannula. The data were analysed with statistical parametric mapping (SPM 94 software from the Wellcome Department of Cognitive Neurology, London) implemented in Matlab (Mathworks, Sherborn, MA). Statistical parametric mapping combines the general linear model (to create the statistical parametric map or SPM) and the theory of gaussian fields to make statistical inferences about regional effect^{27,29}. The scans from each subject were realigned using the first as a reference. Following realignment all images were spatially normalized, using nonlinear transformation, into a standard space and smoothed²⁸. As a final preprocessing step, the images were smoothed using an isotropic gaussian kernel. The condition, subject and covariate effects (global blood flow) were estimated according to the general linear model at each voxel²⁸. To test hypotheses about regionally specific condition effects, the estimates were compared using linear compounds or contrasts. The resulting set of voxel values for each contrast constitute a statistical parametric map of the t statistic, SPMt. The SPMt were transformed to the unit normal distribution (SPMZ) and thresholded at 3.09 (or $P = 0.001$, uncorrected for multiple comparisons).

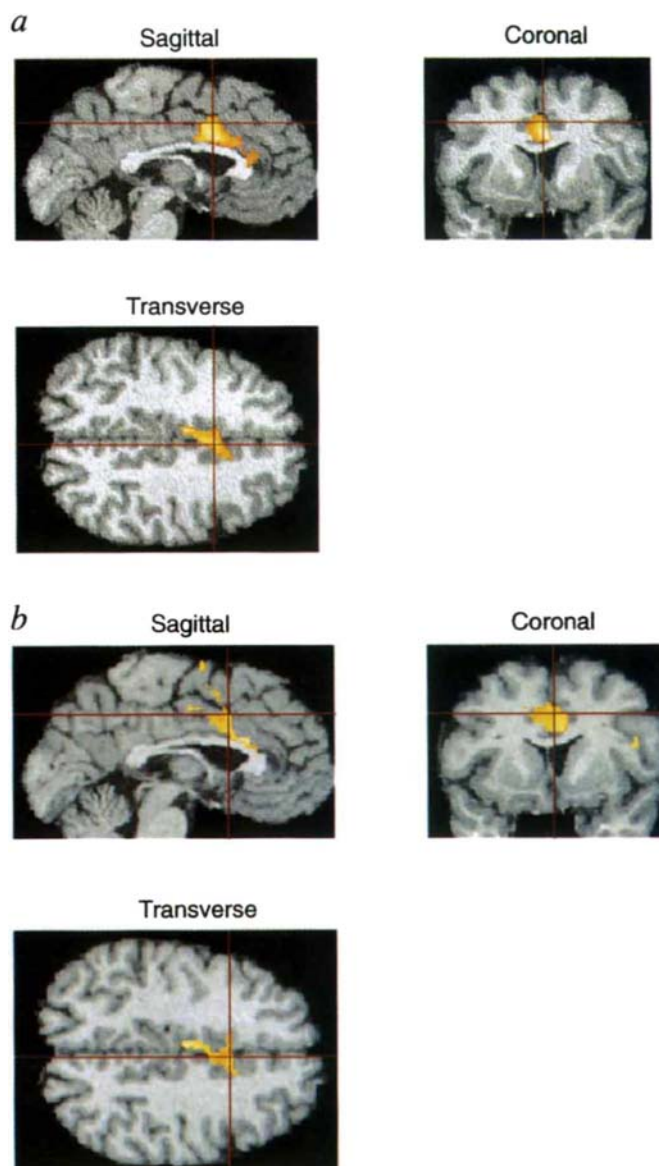
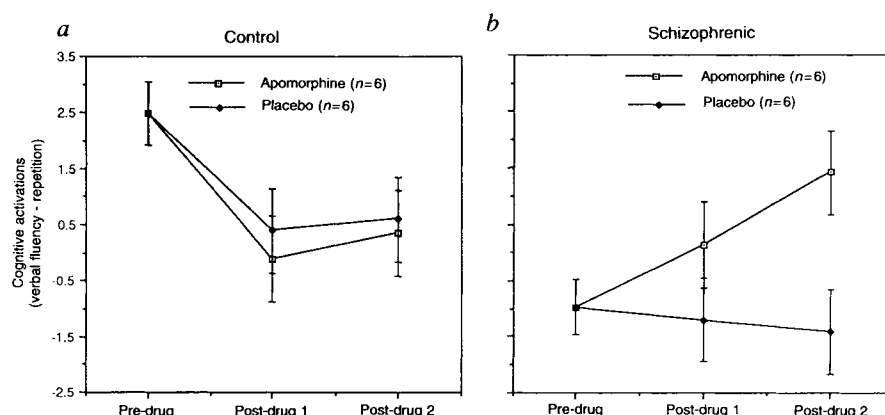


FIG. 3 Relative changes in adjusted rCBF response (adjusted to an arbitrary mean of $50 \text{ ml dl}^{-1} \text{ min}^{-1}$) in the anterior cingulate under cognitive activation (verbal fluency and repetition), pre-drug or post-drug times 1 ($t_1 = 15 \text{ min}$) or times 2 ($t_2 = 30 \text{ min}$). A pixel, at coordinates $x = -4$, $y = -6$, $z = 32$, was chosen on the basis of the focus of maximal difference between controls and schizophrenics under cognitive activation in the non-drug-treated state. This pixel is within a subset of the pixels that define the region where there is a significant difference between controls and schizophrenics post-apomorphine. The data depict responses in the control (a) and schizophrenic (b) groups under cognitive activation at each time point. A relative failure of activation in the schizophrenic patients (averaged response for schizophrenic and control groups) pre-drug is evident. Post-drug, at times t_1 and t_2 , there is a significant augmentation of cingulate activation ($P < 0.001$) in the schizophrenic apomorphine-treated group relative to both control groups and the non-apomorphine-treated schizophrenic group. Error bars were computed on the basis of $\pm 1 \text{ s.e.}$ for the mean effect, and the comparisons are based upon the



interaction term from the SPM. A time-related decrease in the magnitude of activation is evident in both control groups (for extended discussion, see ref. 30). A comparison of the relative decreases post-drug and post-placebo in controls was not statistically significant.

activation on the temporal cortex has been demonstrated in primates²³. A similar effect in humans is implied by a finding that cingulate activation is associated with modulation in a concurrent task-dependent cortical activation²⁴. In the present study we have again observed abnormal fronto-temporal interactions that are modulated post-drug (data not shown). We suggest that in schizophrenia there is a dysregulation in the dopaminergic modulation of cingulate neuronal activity with a consequent impairment in the functional integration of more remote, but anatomically connected, cortical regions. The findings provide a methodology for reconciling neurochemical theories with those that suggest an abnormality in cortico-cortical interactions in schizophrenia. □

Distinct components of spatial learning revealed by prior training and NMDA receptor blockade

D. M. Bannerman*, M. A. Good*, S. P. Butcher†, M. Ramsay & R. G. M. Morris†

Centre for Neuroscience and Department of Pharmacology, University of Edinburgh Medical School, Crichton Street, Edinburgh EH8 9LE, UK

† Fujisawa Institute for Neuroscience, University of Edinburgh, Edinburgh EH8 9JZ, UK

SYNAPTIC plasticity dependent on *N*-methyl-D-aspartate (NMDA) receptors is thought to underlie certain types of learning and memory^{1,3}. In support of this, both hippocampal long-term potentiation and spatial learning in a watermaze are impaired by blocking NMDA receptors with a selective antagonist D(-)-2-amino-5-phosphonovaleric acid (AP5)⁴ or by a mutation in one of the receptor subunits⁵. Here we report, however, that the AP5-induced learning deficit can be almost completely prevented if rats are pretrained in a different watermaze before administration of the drug. This is not because of stimulus generalization, and occurs despite learning of the second task remaining hippocampus dependent. An AP5-induced learning deficit is, however, still seen if the animals are pretrained using a non-spatial task. Thus, despite its procedural simplicity, the watermaze may involve multiple cognitive processes with distinct pharmacological properties; although required for some component of spatial learning, NMDA receptors may not be required for encoding the spatial representation of a specific environment.

After a brief habituation phase, male Lister rats were implanted with minipumps for chronic intracerebroventricular (i.c.v.) infusion of AP5 (30 mM, $0.5 \mu\text{l h}^{-1}$) or artificial cerebrospinal fluid (aCSF) and trained in a watermaze to find a fixed, hidden escape platform (experiment 1). Rats treated with AP5

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* Present addresses: Department of Experimental Psychology, University of Oxford, Oxford OX1 3UD, UK (D.M.B.); School of Psychology, Cardiff University of Wales, Cardiff CF1 3YG, UK (M.A.G.).

† To whom correspondence should be addressed.

failed to learn: they showed no decrease in escape latency over eight training trials scheduled at one trial per day (Fig. 1a), and failed during transfer tests to search within the appropriate quadrant of the pool. (Fig. 1b). In contrast, rats infused with aCSF learned the task readily. All of the rats were subsequently examined for their capacity to show long-term potentiation (LTP) *in vivo* under urethane anaesthesia: AP5 blocked LTP (Fig. 1c). Hippocampal tissue extracts from these same rats showed a mean whole-tissue level of AP5 of 0.75 ± 0.05 nmol per mg wet weight. This reflects an estimated extracellular concentration in hippocampus of $\sim 20 \mu\text{M}$, more than would be required to block NMDA-receptor-dependent LTP *in vivo*⁶.

What is the basis of this apparent learning impairment? It may be due to a contribution of NMDA receptors to spatial learning² (including but not exclusively those mediating some forms of hippocampal LTP), to non-spatial components of learning that are inextricably part of even the simplest watermaze task (such as learning to search for an escape platform, to climb on to it and to treat it as a refuge^{2,7}), or to sensorimotor and/or motivational processes⁸⁻¹¹ necessary for executing the task. We attempted to dissociate these alternatives by systematic variation of an additional pretraining phase scheduled before drug infusion and, in doing so, have found that spatial learning is dissociable into NMDA-receptor-dependent and independent components.

In experiment 2, rats were pretrained in a second watermaze in a separate laboratory ('downstairs'). Minipumps were then implanted and the animals trained exactly as before in the 'upstairs' watermaze. If NMDA receptors are necessary for forming a spatial representation of a new environment, an AP5-induced deficit in learning should still be present.

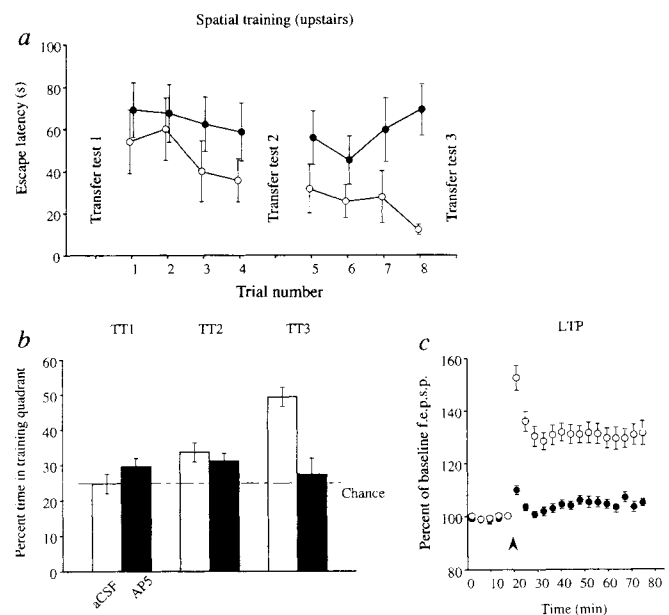
To our surprise, we found that the animals treated with AP5 learned remarkably well. After training downstairs during which

the two groups of rats, which had not yet been treated, did not differ (Fig. 2a), the AP5 group showed a steady decline in escape latency throughout training upstairs. Although the AP5-treated rats were significantly slower than the aCSF group to find the platform (Fig. 2b), which may be due to either a small residual learning impairment or a sensorimotor deficit, both groups searched predominantly in the training quadrant during transfer tests and did not differ statistically (Fig. 2c, d). The rats treated with AP5 again showed a near-complete blockade of LTP *in vivo* (Fig. 2f) and intrahippocampal levels of the drug comparable to those of experiment 1 (0.82 ± 0.05 nmol per mg wet weight). Thus spatial pretraining in a different location has substantially ameliorated the AP5-induced deficit of experiment 1 despite the presence of an apparently complete blockade of LTP *in vivo*.

This new finding would be trivial if there was substantial stimulus generalization between the cues used to navigate in the upstairs laboratory and those used downstairs because AP5 does not affect retention of spatial memory if learning occurs before drug administration in the same environment^{7,12,13}. We had attempted to minimize generalization between the two locations by arranging cues in the room differently and by our escape-platform counterbalancing arrangements (see Fig. 2 legend). Importantly, rats treated with AP5 that were assigned the same platform location downstairs learned successfully to search in different locations upstairs (Fig. 2d). Stimulus generalization can further be ruled out as an explanation because both groups searched at chance during the first transfer test upstairs conducted after training had been completed downstairs (TT1, Fig. 2c), and because both groups remembered the correct pretraining quadrant during a fourth transfer test conducted downstairs after all training had been completed upstairs (TT4, Fig. 2e). Thus training downstairs caused no spatial bias upstairs and

FIG. 1 Experiment 1. a, Escape latency (s) across the 8 trials of training. Animals treated with AP5 (filled circles; 30 mM , $0.5 \mu\text{l h}^{-1}$; $N = 11$) failed to learn (averaging 69.2 ± 12.2 s on trial 8), whereas aCSF rats (open circles; $N = 10$) showed a gradual decline in escape latency (averaging 12.2 ± 2.5 s on trial 8). Analysis of variance (ANOVA) revealed a significant effect of groups ($F = 6.86$, d.f. 1/19, $P < 0.025$). b, Percentage time spent in the training quadrant of the pool during the transfer tests (TT1–3). ANOVA of TT3 showed significant effects of quadrants ($F = 12.15$, d.f. 2/57, $P < 0.0001$; numerator d.f. reduced by 1 as quadrants percentage times necessarily add to 100%), and groups $\times$ quadrants ($F = 7.16$, d.f. 2/57, $P < 0.005$). A separate ANOVA of percentage time in the training quadrant showed the significant superiority of the controls ($49.4 \pm 2.8\%$) over the AP5-treated rats ($27.4 \pm 4.6\%$; $F = 15.79$, d.f. 1/19, $P < 0.001$) who did not differ from chance. c, The aCSF rats showed $130.5 \pm 4.2\%$ LTP *in vivo* 50–60 min after high-frequency activation of the perforant path. LTP was blocked in the AP5 group ($104.3 \pm 1.6\%$).

METHODS. Behavioural training and surgery. Male Lister hooded rats were habituated to the watermaze by giving them 6 swimming trials with heavy white curtains surrounding the pool to occlude extramaze cues. They were then stereotactically implanted under Avertin anaesthesia with an i.c.v. cannula connected to an Alza 2002 minipump containing either AP5 (30 mM) or aCSF. Three days later, they were trained in a 2-m diameter open-field watermaze containing opaque water at $25 \pm 1^\circ\text{C}$ in a laboratory containing prominent cues including a section of primate caging, wall posters and a set of white curtains hanging at the edge of the pool. The ceiling of the room was illuminated by four 500 W lamps and the paths taken by the rats within the pool monitored with an overhead video camera connected to a fully automated computer tracking system. The escape platform (11 cm diameter) was placed in either the northeastern or southwestern quadrant (counterbalanced within each group). The training protocol began with a transfer test (in which the platform was absent and the rats allowed to swim in the pool for 60 s), followed immediately by the first trial of training (platform present). Training continued at 1 trial per day, with transfer test 2 conducted just before the 5th daily trial, and transfer test 3 on the day after the 8th training trial. Electrophysiology. Following transfer test 3, all rats were anaesthetized with urethane (1.5 gm per kg) and stereotactically implanted with a bipolar stimulating electrode



in the perforant path and a monopolar recording electrode in the hilus of the dentate gyrus. Low-frequency test pulses (0.05 Hz , $700 \mu\text{A}$) were given to find a positive going dentate field potential. When the early rising slope of this potential had been stable for at least 60 min, the rats were given 4 trains of 33 pulses at 250 Hz at 10-s intervals followed by a return to low-frequency stimulation. Potentials were then monitored for 1 h. The animals were then killed with Euthatal, the brains removed and tissue samples from the left and right hippocampus were dissected out and stored at -20°C for later high-performance liquid chromatography (HPLC) analysis of AP5 content²⁷.

memory of where to search downstairs survived intervening training upstairs.

A second concern was that spatial pretraining might reduce the necessity for hippocampal involvement in spatial learning in a new environment. To investigate this possibility, in experiment 3, further animals were trained in the same spatial pretraining task downstairs and were then given either ibotenic acid lesions of the hippocampus¹⁴, sham surgery, or left unoperated, and trained on exactly the same spatial learning task upstairs. A lesion-induced deficit in escape latency (data not shown) and transfer test performance (Fig. 2g) was clearly apparent. Spatial learning remains hippocampus-dependent after previous training in a similar task.

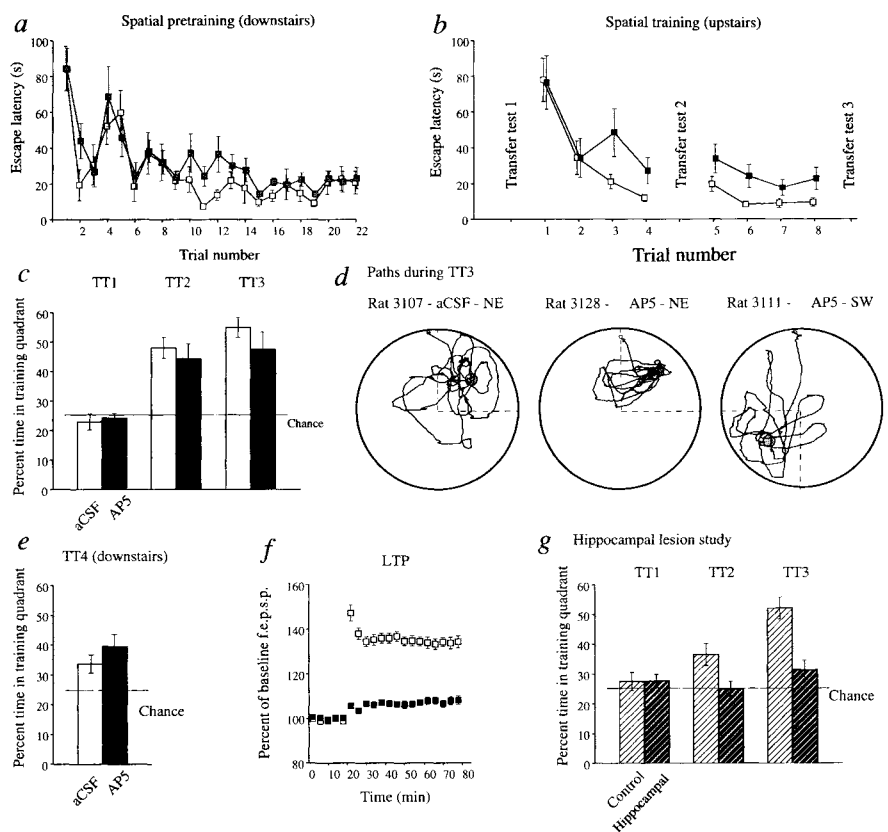
Thus, the results of experiment 2 are apparently inconsistent with the hypothesis that NMDA receptors are necessary for hippocampus-dependent spatial learning². The different outcomes of experiments 1 and 2 also rule out an account of the deficit of experiment 1 exclusively in terms of a direct drug-induced disruption of sensorimotor or motivational processes,

although this might contribute to the small residual impairment in escape latency found in experiment 2. The implication seems to be that disrupting NMDA receptors interferes with the non-spatial procedural learning¹⁵ that is intrinsic to acquisition of knowledge of the watermaze (such as learning to use the platform as a refuge). Pretraining downstairs would enable this learning to occur normally and, assuming some transfer between tasks, reduce the drug-induced deficit in the second task.

However, if this interpretation is correct, pretraining downstairs need not be a spatial task that is essentially identical to that later used upstairs. Non-spatial pretraining should suffice to ameliorate the usual AP5 deficit in learning the watermaze. To test this inference, experiment 4 explored the effect of pretraining that minimized the opportunity for spatial learning. Curtains were drawn around the downstairs pool to obscure extraneous cues and the platform was hidden in a different location on each trial. This resulted in longer escape latencies downstairs (Fig. 3a) but, contrary to the prediction, an AP5 deficit in learning the upstairs task reappeared. The AP5 group was

FIG. 2 Experiments 2 and 3. **a**, Escape latency (s) across the 22 trials of pretraining in the downstairs watermaze by rats later to be given aCSF (open squares) or AP5 (shaded squares). Escape latency averaged 16.2 ± 2.7 s by the end of this spatial pretraining (trials 19–22). **b**, Escape latency (s) across the 8 trials of training in the upstairs watermaze. Both AP5 (black squares, 30 mM, $N=11$) and aCSF (open squares, $N=13$) rats showed a striking reduction in escape latency across trials ($F=16.93$, d.f. 7/154, $P<0.0001$), although the groups effect was still significant ($F=4.59$, d.f. 1/22, $P<0.05$; the AP5 group took 22.7 ± 6.2 s and the aCSF group averaged 9.5 ± 2.4 s on trial 8). **c**, Percentage time in the training quadrant only during transfer tests 1–3 (chance=25%). The groups did not differ at either transfer test 2 or 3 (TT3: $F=1.27$, d.f. 1/22, $P>0.25$), but both showed a strong bias towards searching in the training quadrant ($F=42.8$, d.f. 2/66, $P<0.0001$). **d**, Paths taken by representative rats in the aCSF and AP5 groups in TT3. The two AP5 rats were trained to the same platform location downstairs, but to different locations upstairs, showing that spatial learning has occurred in the presence of the drug. **e**, Percentage time in the training quadrant only during transfer test 4 conducted downstairs after completion of training upstairs. Both aCSF and AP5 groups are above chance in remembering the correct downstairs training quadrant despite half the rats in each group having been trained to the two different locations upstairs. **f**, Mean percentage slope potentiation of dentate field excitatory postsynaptic potential (f.e.p.s.p.) showing near-complete blockade of LTP in the AP5-treated group. **g**, Percentage time in the training quadrant only during the transfer tests by control (sham, $N=8$, unoperated, $N=8$, combined, because they did not differ, into a single control group) and hippocampal lesioned ($N=13$) rats from experiment 3 ($F=16.96$, d.f. 1/27, $P<0.0005$). The performance of the lesioned animals did not differ from chance despite their having had 22 trials of spatial pretraining downstairs before the lesion and 8 trials after the lesion upstairs.

METHODS. Behavioural training, surgery and electrophysiology for experiment 2. Following the same habituation day as used in experiment 1, male Lister hooded rats were trained in a second watermaze of identical shape and size located in a different laboratory with distinctively different cues (the 'downstairs' watermaze). The cues in the room included an animal rack covered with black plastic, no visible hanging curtains, and different positions of the access door and computer system from those 'upstairs'. The animals were trained to an escape platform which was consistently in the same location for all 22 training



trials as follows: for each set of 4 animals subsequently trained upstairs to either northeast or southwest, the platform downstairs was located in the northeast for one rat, and in the northwest, southwest or southeast for the others. The number of pretraining trials per day was 4, 4, 4, 2, 2, 1, 1, 1 and 1. The animals were then implanted with minipumps containing AP5 (30 mM) or aCSF and then trained in the upstairs watermaze according to exactly the same protocol as used for experiment 1. The LTP and HPLC assays followed as before. Experiment 3. The behavioural training was identical to experiment 2 in the habituation, downstairs pretraining and upstairs training phases. The only differences were that, instead of implanting minipumps, the rats were given lesions of the hippocampus¹⁴ and allowed 10 days postoperative recovery. Minute quantities of ibotenic acid (0.05–0.10 μ l) were given at each of 28 injection sites using stereotaxic coordinates adapted for Lister hooded rats. At the end of training, the lesions were assessed using thionin-stained sections. All lesions were between 80 and 100% of total hippocampal volume with minimal extra-hippocampal damage.

highly significantly impaired with respect to escape latency (Fig. 3b) and during both transfer tests 2 and 3 (Fig. 3c, d). Rats treated with AP5 again showed a near-complete blockade of LTP *in vivo* (Fig. 3e), and comparable whole-tissue levels of the drug in hippocampus (0.68 ± 0.06 nmol per mg wet weight). Thus non-spatial pretraining fails to prevent an AP5-induced impairment of spatial learning with respect to both escape latency and spatially localized searching during the transfer tests, although this deficit not as severe as that shown by animals given no pretraining.

These experiments were conducted successively, and statistical comparisons between them should be treated with caution. However, a comparison of percentage time in the training quadrant in transfer tests 2 and 3 of experiments 1, 2 and 4 indicated a significant triple interaction between drug group, pretraining condition, and transfer test ($F = 4.23$, d.f. 2/64, $P < 0.025$; Fig. 4). Pretraining improved the performance of rats treated with aCSF and with AP5. The basis of the triple interaction is that, with no pretraining, rats treated with AP5 showed no evidence of learning over eight trials, non-spatial pretraining allowed an intermediate amount of learning with impaired but above-chance performance by transfer test 3, and spatial pretraining largely ameliorated the learning deficit normally induced by AP5 within as few as four trials.

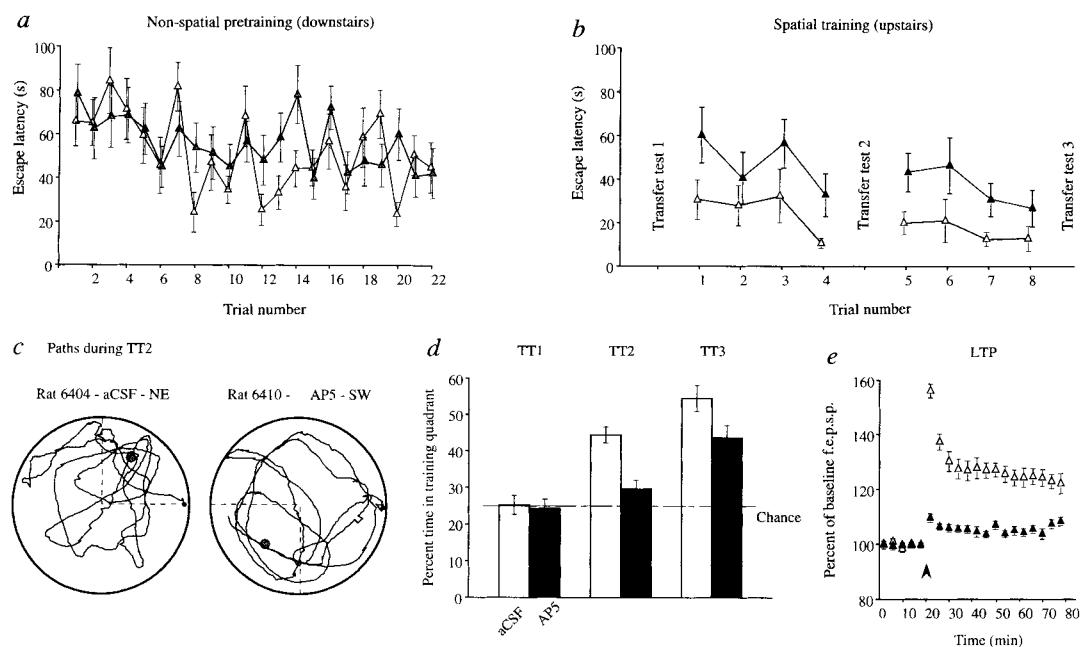
Systematic comparison of training protocols involving no pretraining, non-spatial pretraining, or spatial pretraining has indicated varying effects of an NMDA receptor antagonist on subsequent spatial learning. If disrupting NMDA receptors affected only the spatial components of watermaze learning, animals given non-spatial pretraining (experiment 4) should have learned no better than those given no pretraining (experiment 1). Similarly, if NMDA receptor antagonism affected only

the non-spatial components, the rats given spatial pretraining (which also enables these non-spatial components to be acquired) (experiment 2) should have learned no better than those given non-spatial pretraining (experiment 4). Neither outcome occurred. The results indicate, therefore, that chronic i.c.v. AP5, at a level in excess of that required to block LTP *in vivo* under urethane anaesthesia, probably disrupts both spatial and non-spatial components of watermaze learning. We cannot rule out the alternative possibility that a spatially diffuse search strategy is acquired during non-spatial pretraining which, with AP5 treatment, can only gradually be inhibited. That is, the different outcomes of experiments 2 and 4 might reflect a general inflexibility of learned behaviour under the influence of AP5 which should be particularly apparent during reversal learning. Nonetheless, the pattern of results indicates the importance of recognizing that the procedural simplicity of the watermaze task belies its underlying psychological complexity. Several potentially dissociable learning processes are involved, possibly depending on the interaction of different cerebral circuits^{16, 19}.

It is possible that spatial learning can be divided into pharmacological dissociable components. One of these, forming a memory representation of the spatial layout of an environment²⁰, appears to be less critically dependent on NMDA receptors than argued previously^{2, 4}, because learning the new upstairs environment was unaffected by AP5 in animals given previous spatial training. Although non-NMDA-dependent synaptic plasticity²¹ may be involved in this component of spatial learning, it should be noted that this new result conflicts with other findings obtained using a radial maze¹³, possibly because of subtle differences in task requirements (working memory versus reference memory). The discrepancy should be explored further using single-unit recording techniques²² and other learning procedures

FIG. 3 Experiment 4. a, Escape latency (s) across the 22 trials of non-spatial pretraining in the downstairs watermaze by the two groups subsequently treated with aCSF and AP5 (aCSF, open triangles; to-be-AP5, grey triangles). The mean escape latency over the last 4 trials was 47.2 ± 3.0 s. Compare with the spatial pretraining of Fig. 2a. b, Escape latency (s) in the upstairs watermaze. Both AP5 (filled triangles, $N = 13$) and aCSF (open triangles, $N = 12$) rats show a reduction in escape latency to 26.8 ± 8.4 s and 12.8 ± 5.8 s, respectively, reflecting significant trials ($F = 2.55$, d.f. 7/161, $P < 0.05$) and groups ($F = 10.62$, d.f. 1/23, $P < 0.005$) effects. c, Paths taken during transfer test 2 by representative animals in the aCSF and AP5 groups. Note the relatively focused searching by the aCSF rat after only 4 training trials in the upstairs watermaze, but near-random searching by the AP5 rat. d, Percentage time in the training quadrant only during the transfer tests (chance = 25%). The groups differed strikingly at TT2 (aCSF = $44.4 \pm 2.2\%$; AP5 = $29.6 \pm 2.5\%$; $F = 19.80$, d.f. 1/23, $P < 0.0001$) and still significantly at TT3 (aCSF = $54.5 \pm 3.6\%$; AP5 = $43.7 \pm 3.4\%$; $F = 4.89$, d.f. 1/23, $P < 0.05$). e, Mean percentage slope potentiation of dentate f.e.p.s.p. showing near-complete blockade of LTP in the AP5-treated group.

METHODS. Behavioural training, surgery and electrophysiology for



experiment 3. Following the same habituation day as used in experiment 1, male Lister hooded rats were trained as normal animals in a non-spatial pretraining task in the downstairs watermaze. The pool was surrounded by curtains to occlude extramaze cues and the animals trained to an escape platform which was in a different location for each of the 22 training trials (the number of trials per day was 4, 4, 4, 2, 2, 2, 1, 1, 1 and 1 as in experiment 2). The rats were then implanted with minipumps containing AP5 (30 mM) or aCSF and, two days later, were trained in the upstairs watermaze according to exactly the same protocol as experiments 1 and 2 (half of each group being trained to either northeast or southwest). The LTP and HPLC assays followed as before.

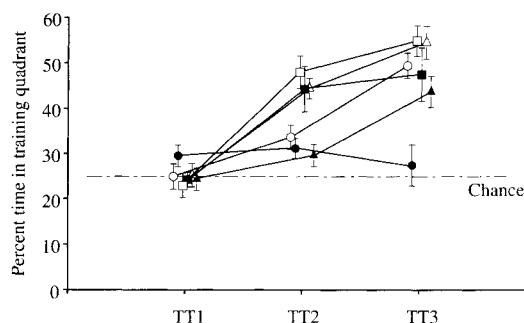


FIG. 4 Comparison of the percentage time in the upstairs training quadrant ($\pm$ s.e.m.) in transfer tests 1, 2 and 3 by the three AP5 (filled symbols) and three aCSF (open symbols) groups given either no pre-training downstairs (circles, experiment 1), spatial pretraining (squares, experiment 2) or non-spatial pretraining (triangles, experiment 4) (chance = 25%). Note all groups started upstairs at chance levels of performance. As noted in the text, the ANOVA revealed a significant triple interaction between drug group, pretraining condition, and transfer test ($F = 4.23$, d.f. 2/64, $P < 0.025$). Non-pretrained AP5-treated rats showed no evidence of spatial learning at any point. AP5-treated rats given non-spatial pretraining showed little spatial learning at TT2 but substantially more than the non-pretrained AP5 group by TT3 ($P < 0.01$; planned F -test using error-mean square from ANOVA). AP5 rats given spatial pretraining learned equivalently to their aCSF controls ($P > 0.10$) and showed clearly better performance than the non-spatially pre-trained AP5 group during both transfer test 2 ($P < 0.05$) and transfer test 3 ($P < 0.05$).

that may be more sensitive to disruption of LTP²³. Other components of spatial learning could include learning to attend appropriately²⁴, to navigate in a spatially directed way²⁵, and/or to develop expectations about the reliable location of fixed objects in space²⁶. Such learning cannot take place during non-spatial pretraining. If acquiring this information usually involved NMDA-receptor dependent synaptic plasticity, and was dependent upon the hippocampus for its expression (even if stored elsewhere), a dissociation between the effects of hippocampal lesions and AP5 upon subsequent spatial learning would result (experiments 2 and 3). This is precisely what we observed in animals given spatial pretraining. □

Spatial learning without NMDA receptor-dependent long-term potentiation

Deborah Saucier & Donald P. Cain

Department of Psychology and Graduate Program in Neuroscience, University of Western Ontario, London, Ontario N6A 5C2, Canada

HIPPOCAMPAL lesions impair spatial learning in the watermaze¹. Drugs that antagonize *N*-methyl-D-aspartate (NMDA)-receptor activity, which is required for long-term potentiation (LTP) at various hippocampal synapses²⁻⁴, block LTP and impair water-maze learning⁴⁻⁶. This has led to the hypothesis that NMDA receptors, through their involvement in LTP, may be necessary for spatial⁴⁻¹¹ and other forms of learning^{12,13}. We examined this hypothesis using NPC17742 (2*R*,4*R*,5*S*-2-amino-4,5-(1,2-cyclohexyl)-7-phosphonoheptanoic acid), a potent and specific antagonist of NMDA receptors¹⁴⁻¹⁷. Here we report that NPC17742 completely blocked dentate gyrus LTP but did not prevent normal spatial learning in rats that had been made familiar with the general task requirements by non-spatial pretraining. Although these results do not rule out a contribution of NMDA-mediated dentate LTP to spatial learning, they indicate that this form of LTP is not required for normal spatial learning in the watermaze.

In experiments that involved a detailed behavioural analysis of watermaze learning in naive rats given NMDA antagonists, performance of the required behaviours was impaired by the sensorimotor disturbances that result from NMDA antagonism^{18,19}, such as thigmotaxis and slowed swimming, or swimming over or deflecting off the platform. Correlations between the sensorimotor disturbances and measures of maze acquisition were robust. Non-spatial pretraining^{5,6} in the general task requirements eliminated acquisition deficits^{18,19}. Therefore we used non-spatial pretraining to evaluate whether rats familiar with the task requirements could acquire spatial information when NMDA-dependent LTP was blocked. The same rats were used in both electrophysiological and behavioural testing to confirm that LTP was blocked by the same treatment as used for NMDA receptor antagonism during maze testing. Groups and procedures are summarized in Fig. 1.

We first evaluated the ability of NPC17742 (5.0 mg per kg body weight in dimethyl sulphoxide, intraperitoneal) to block dentate gyrus LTP. NPC17742, a potent and specific competitive NMDA-receptor antagonist^{14,17}, readily crosses the blood-brain barrier. The dose used was twice the 50% effective dose for blocking NMDA-induced seizures and had the expected effects on seizures, spontaneous behaviour, motor ability^{14,19} and kindling (D.S. and D.P.C., manuscript submitted). NPC17742 completely blocked the induction of LTP (Fig. 2).

One subgroup ($N = 7$) of pretrained NPC-treated rats was trained in the watermaze, followed 1–2 weeks later by LTP without NPC17742. The other subgroup ($N = 7$) was tested similarly in reversed order. Three rats failed to complete this LTP test, leaving subgroups of 5 and 6 rats, which did not differ on any measure ($P > 0.05$) and were combined. Without NPC17742 the expected LTP occurred (Fig. 2), confirming that the experimental arrangements were capable of inducing and detecting LTP.

Five days after non-spatial pretraining, the pretrained NPC-treated rats received 5.0 mg per kg NPC17742, and 1 h later were trained in the hidden-platform task (10 trials; elapsed time 1 h) followed by a 1-min probe trial (hidden platform removed), followed by the visible-platform task (10 trials). Other groups also completed the watermaze task (Fig. 1).

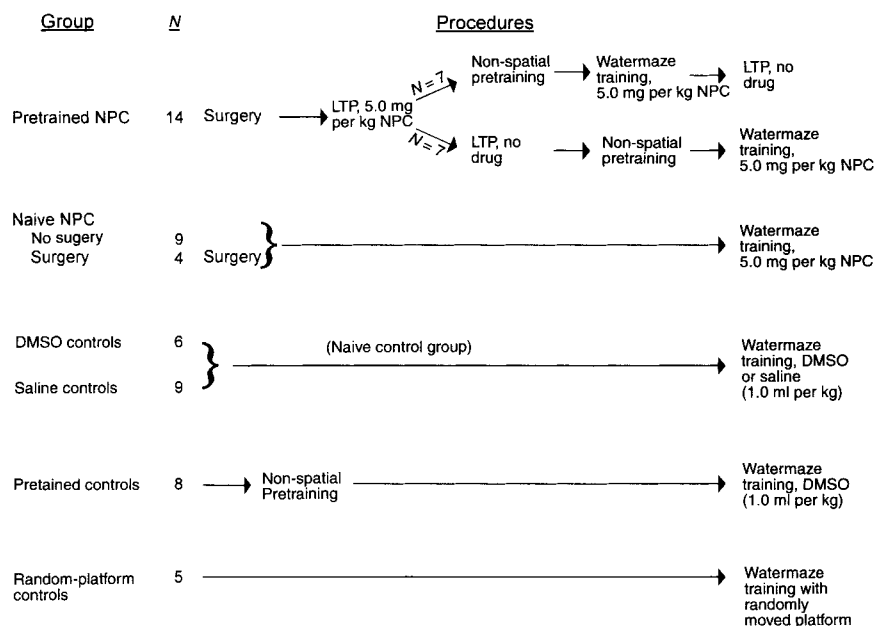
The improvement in mean search time for the pretrained groups from pretraining days 1–4 indicated that they acquired

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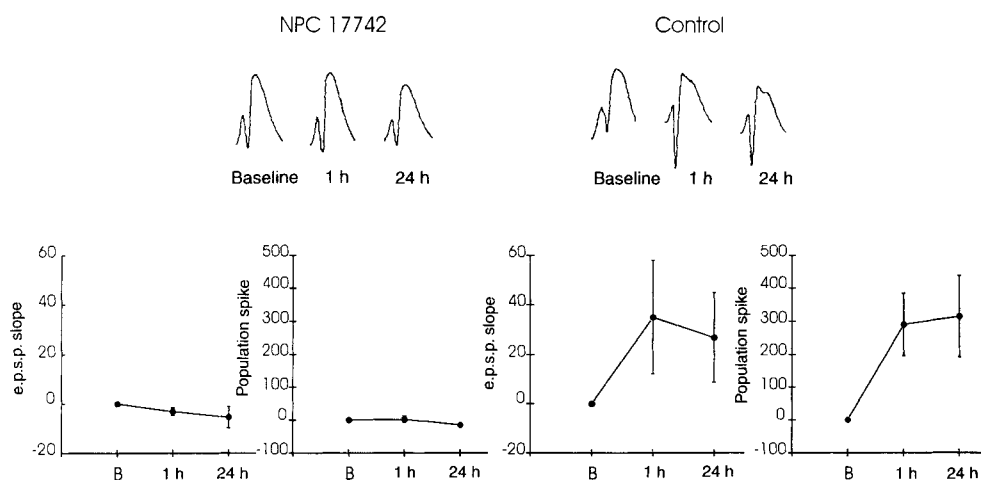
FIG. 1 Flow diagram indicating groups, procedures and treatments. *N* indicates number of subjects. Four naive NPC-treated rats with electrode implants to evaluate effects on maze learning did not differ from the unimplanted subgroup on any measure ($P > 0.05$). DMSO and saline controls did not differ on any measure ($P > 0.05$) and were combined into the naive control group. Surgery and LTP procedures were similar to those in earlier reports^{24,28} and are described in Fig. 2. For watermaze training a white circular (1.5 m) pool with a hidden platform (15 × 15 cm) 1 cm below the water surface was used. Water temperature was 29 °C, and animals were placed under a heat lamp between trials. Swim paths were videotaped, digitized, and analysed using the Poly-Track system (San Diego Instruments). For visible-platform training trials the platform protruded 2.5 cm above the surface, was marked by a 15-cm high object, and was moved between trials. Non-spatial pretraining for those rats that received it was conducted on 4 consecutive days beginning 8 days before water-maze training began (3 trials per day, 4-h inter-trial interval, 12 trials in total, no drug administered), with the hidden platform moved pseudorandomly to a new quadrant after every trial^{5,6,18,19}. Black curtains around the pool eliminated distal cues. Rats swam until they found the hidden platform or until 120 s elapsed, at which time they were placed on the platform, where they remained for 30 s.



strategies appropriate for coping with the task, such as swimming away from the maze wall (pretrained NPC group, 44.0, 19.2 s; pretrained controls, 42.8, 19.0 s). The pretrained NPC group readily learned the location of the hidden platform, with search times similar to those of the naive and pretrained control groups (Fig. 3a). Analysis of behaviour in the pool revealed numerous sensorimotor disturbances in the naive NPC group, but few disturbances in the pretrained NPC group¹⁹ (data not

shown). Similar results occurred in the visible-platform task; the naive NPC group had longer search times than the pretrained NPC group (Fig. 3a), which is consistent with sensorimotor disturbances interfering with performance of this cue-mediated task^{19,20}. Probe trial search time in the hidden-platform quadrant remained near chance (15 s) for the naive NPC and random-platform groups but above chance for the other groups (Fig. 3b), suggesting that they had learned the spatial location of the

FIG. 2 Effects of NPC17742 on LTP (left), and normal LTP (no NPC17742, right) in the pretrained NPC group. Representative averages at baseline and at 1 and 24 h after high-frequency trains are from the same rat (top). Group mean ($\pm$ s.e.m.) values (bottom) represent the baseline (B), and percentage change at 1 and 24 h. One-tailed non-parametric Wilcoxon signed-ranks tests were used to evaluate the reliability of the changes because the data were not normally distributed, and we predicted that under normal conditions the measures would increase^{24,28}. Baseline measures did not differ between the NPC17742 and no-treatment conditions ($P > 0.05$), indicating good stability of the experimental arrangements. The measures did not change ($P > 0.05$) under NPC17742 (left). The excitatory post-synaptic potential (e.p.s.p.) slope increase in the no-treatment condition (right) was reliable at 1 h ($P < 0.025$) and 24 h ($P < 0.05$); the population spike increase was reliable at 1 h ($P < 0.005$) and 24 h ($P < 0.005$). Male hooded rats anaesthetized with sodium pentobarbital (65 mg per kg) received implantation of chronic stimulating and recording electrodes (127 μ m) into the perforant path and the ipsilateral dentate hilus to record evoked field potentials^{24,28}. Single biphasic (0.1 ms per phase) test pulses were delivered only during behavioural immobility²⁸, a minimum of 10 s apart. Final positioning of the electrodes was determined by recordings from the hilar electrode. Responses were digitized,



averaged (10 sweeps), and analysed for the rising phase of the field e.p.s.p. and area under the population spike. Rats studied exhibited a negative-going extracellular population spike to stimulation $<100 \mu$ A; the response to a test pulse of 800 μ A was 10.4 ± 1.3 mV (mean $\pm$ s.e.m.). Input/output determination was followed by NPC17742 and determination of baseline 1 h later. For LTP, 10 high-frequency trains (8 biphasic pulses, 0.1 ms per phase, 400 Hz, near-maximal input/output response) were applied to the perforant path. Rats were unanaesthetized throughout testing.

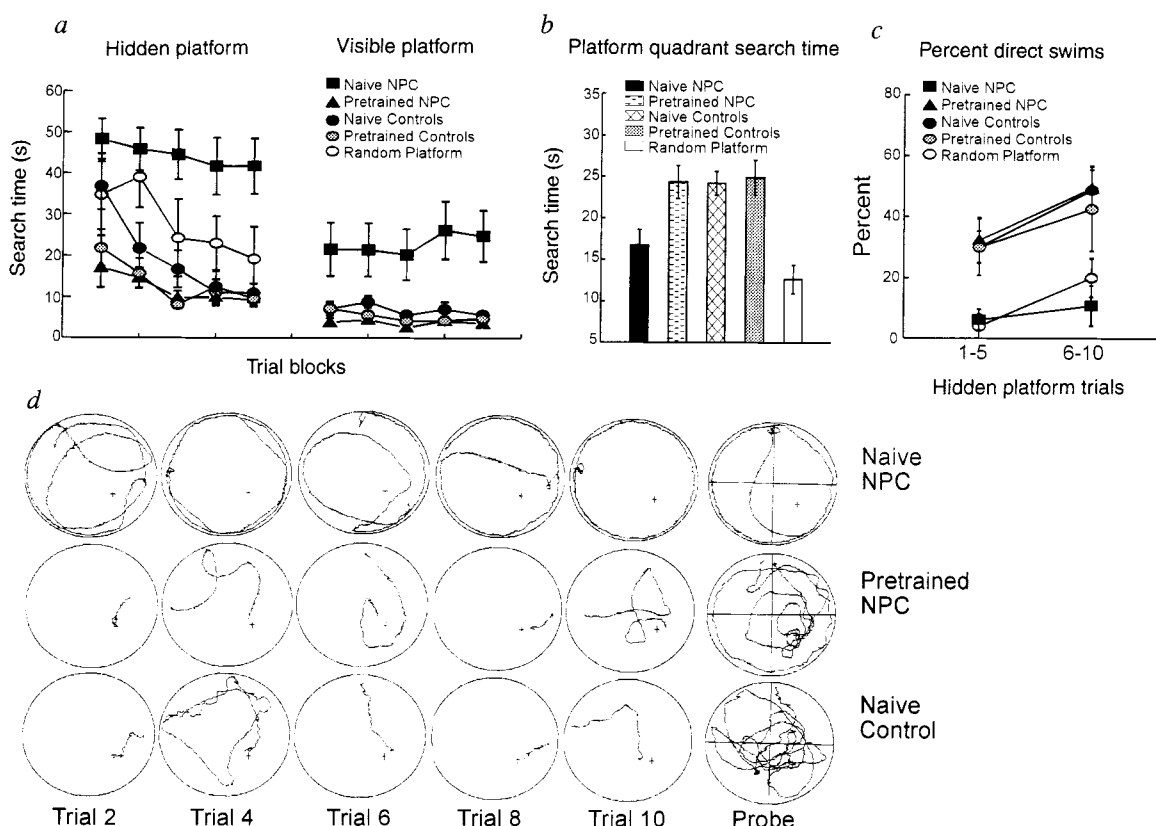


FIG. 3 Watermaze acquisition. *a*, Mean ($\pm$ s.e.m.) search times (s) for hidden and visible platform tasks plotted as 2 trials per block. Repeated measures ANOVA of hidden-platform data yielded effects of group ($F(3,9)=18.1$, $P<0.0001$; naive NPC versus all other groups, $P<0.05$, Newman-Keuls), trials ($F(9,405)=11.8$, $P<0.0001$), and a group $\times$ trials interaction ($F(27,405)=1.5$, $P<0.05$). Separate repeated-measures ANOVAs of pretrained NPC, pretrained control, or naive control data yielded block effects, indicating comparable learning in these groups (pretrained NPC, $F(4,44)=3.1$, $P<0.03$, block 1 versus 3, 4 or 5, and block 2 versus 5, $P<0.05$; pretrained control, $F(4,24)=6.0$, $P<0.002$, block 1 versus 3, 4 or 5, and block 2 versus 3, $P<0.05$; naive control, $F(4,52)=9.5$, $P<0.001$, block 1 versus 2, 3, 4 or 5, and block 2 versus 5, $P<0.05$). Analysis of visible-platform data yielded an effect of group ($F(3,9)=7.6$, $P<0.001$; naive NPC versus all other groups, $P<0.05$; pretrained NPC versus naive control group, $P<0.05$). These findings were robust; for data in *a*–*c*, when the 3 pretrained NPC rats that did not complete the no-treatment LTP procedure and the 4 naive NPC rats with electrode implants were excluded from the analysis, the identical

pattern of findings was obtained. *b*, Mean search time in the hidden-platform quadrant after training. Search time differed among the groups ($F(3,34)=4.4$, $P<0.008$; naive NPC versus all other groups, $P<0.05$). *c*, Mean percentage direct swims (a swim path that remained inside a band 18cm wide from the start to the hidden platform). Analysis as in *a* yielded effects of group ($F(3,1)=9.0$, $P<0.0001$; naive NPC versus all other groups, $P<0.05$) and trials ($F(1,45)=8.4$, $P<0.006$). *d*, Individual swim paths and probe trials for rats with the summed hidden-platform search time closest to their group mean. Cross indicates hidden-platform position. Naive NPC rats swam thigmotactically and infrequently encountered the hidden platform. Pretrained NPC and naive control rats searched the inner areas of the pool, frequently encountering the hidden platform; having missed the platform they swam back to it (pretrained NPC, trials 6, 10; naive control, trial 4). Pretrained control rats swam similarly (not shown). Search time in the hidden-platform quadrant (probe trial) for these 3 rats was: naive NPC, 12.4 s; pretrained NPC, 24.0 s; naive control, 20.2 s.

hidden platform. The percentage of direct swims was similar in the pretrained NPC, pretrained control, and naive control groups (Fig. 3c), a further indication of spatial learning. Naive NPC rats swam thigmotactically and seldom encountered the platform, whereas the other groups searched the inner area of the pool, frequently encountering the platform (Fig. 3d).

In two earlier studies involving non-spatially pretrained rats trained under NMDA antagonism with D(-)-2-amino-5-phosphonovaleric acid (AP5), the pretraining improved but did not completely normalize watermaze performance^{5,6}. Our consistent finding in this and earlier research^{18,19} has been that non-spatially pretrained rats can learn the watermaze as effectively as controls. Two differences in procedure, among others^{18,19}, might explain the different results. First, the substantially greater task difficulty and longer mean intertrial interval in the earlier studies^{5,6} would be expected to prevent the AP5-treated rats from finding the hidden platform as effectively as controls within the limited number of trials allowed²¹. Second, our use of an acute drug administration and behavioural testing protocol avoided possible

disruptive consequences on maze learning of the pathomorphological reaction that occurs in neocortex in response to chronic administration of NMDA antagonists^{22,23}.

The occurrence of robust spatial learning under the same NMDA antagonism that blocked LTP suggests that NMDA-mediated dentate-gyrus LTP is not required for spatial learning in this task, a conclusion consistent with reports that LTP saturation does not disrupt spatial learning^{24–27}. The possibility that NMDA-mediated LTP makes a non-essential contribution to spatial learning cannot be excluded, but the demonstration of this will require additional research. □

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NT-4-mediated rescue of lateral geniculate neurons from effects of monocular deprivation

David R. Riddle, Donald C. Lo & Lawrence C. Katz

Department of Neurobiology, Duke University Medical Center, Durham, North Carolina 27710, USA

ALTERING the balance of activity between the two eyes during the critical period for visual-system development profoundly affects competitive interactions among neurons in the lateral geniculate nucleus and primary visual cortex^{1–6}. Neurons in the lateral geniculate nucleus that are deprived of activity by closing or silencing one eye atrophy as a result of competition with non-deprived neurons for some critical factor(s) presumed to be present in the cortex. Based on their actions in the developing visual system^{7–12}, neurotrophins are attractive candidates for such factors. We tested whether neurotrophins mediate intracortical competition of afferents from the lateral geniculate nucleus by using monocular deprivation and a new method for highly localized, *in vivo* delivery of neurotrophins. This method allowed unambiguous identification of neurons that were exposed to neurotrophin. Here we report that only one neurotrophin, the TrkB ligand NT-4, rescued neurons in the lateral geniculate nucleus from the dystrophic effects of monocular deprivation.

To overcome limitations of common methods for *in vivo* application of neurotrophins (such as osmotic minipumps and intracortical or intraventricular injection) we developed a new delivery system in which neurotrophins were bound to fluorescent latex microspheres. Coated microspheres had activity comparable to that of free neurotrophin, retained activity for at least four days at 37 °C (ref. 13), and remained competent to undergo retrograde axonal transport. To determine whether neurotrophins modulate the effects of monocular deprivation (MD) in the developing visual system, we closed one eye of each of 21 ferrets (postnatal day 38 to 42) and made injections of microspheres coated with nerve growth factor (NGF), NT-4, NT-3 or brain-derived neurotrophic factor (BDNF) into the contralateral primary visual cortex (V1; Fig. 1, top). The microspheres were taken up by nerve terminals and retrogradely

transported¹⁴. Thus we could identify the projection neurons in the lateral geniculate nucleus (LGN) that were exposed to neurotrophin (Fig. 1, middle and bottom). Within individual animals, adjacent injections of control microspheres allowed comparison of these neurons with others that projected to V1

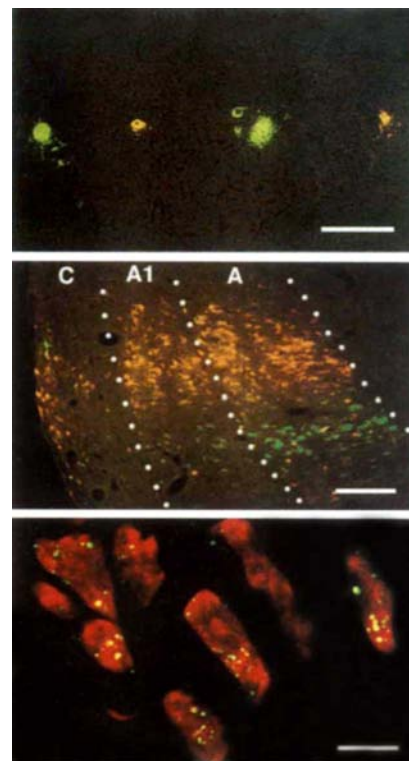


FIG. 1 *In vivo* delivery of neurotrophins. A horizontal section through area 17 of ferret cortex is shown containing four injection sites, two with red NT-4-coated microspheres (which appear orange in this double exposure) and two with green control microspheres (top; scale bar, 500 μm). Columns of retrogradely labelled neurons extend across the A and A1 layers of the LGN after injections in V1 (middle; scale bar, 200 μm); laminar borders are indicated by dotted lines (sublaminae are apparent within A and A1). Adjacent regions of red and green labelled cells projected to adjacent injection sites in the visual cortex. At higher magnification, green microspheres are apparent throughout the cytoplasm of several labelled neurons (bottom; scale bar, 10 μm, dual-channel confocal micrograph).

METHODS. Ferrets (P38–P42) were prepared for aseptic surgery as previously described²⁸. A burr hole was opened on the right side to expose much of the length of area 17, which in the ferret lies at the posterior pole of the cortex²⁹. The dura was incised and microspheres were pressure injected at 4–6 sites in V1 (in the area corresponding to central visual field), using a glass micropipette with a tip diameter of 20–30 μm. For each injection the pipette was advanced to a depth of 0.85 mm and approximately 200 nl of microspheres was then ejected as the pipette was withdrawn. Injections of neurotrophin-coated and control microspheres of different colours were interdigitated and separated by 0.75–1.0 mm (both immunocytochemical analysis with antibodies to NGF and injections of horseradish peroxidase-coated microspheres indicated that proteins do not diffuse from the microsphere injection site). The contralateral eye was then closed using standard techniques¹. One litter of ferrets was used to test the effects of each neurotrophin; in half of the ferrets from each litter the neurotrophin was bound to red microspheres and the controls were green; in the remainder the colours were reversed. The ferrets were killed after four days by sodium pentobarbital overdose (100 mg kg⁻¹) and perfused with phosphate-buffered paraformaldehyde. The brain was removed, blocked, postfixed and cryoprotected. The right occipital cortex was dissected for separate sectioning before the block containing the right LGN was sectioned in the sagittal plane using a cryostat. Alternate sections (30 μm) of the LGN were mounted, counterstained with ethidium bromide, and coverslipped.

but were not exposed to neurotrophin. We verified that brief MD, like prolonged MD¹⁶, caused shrinkage of neurons in the closed-eye layer of the LGN, and then we determined whether neurons that accumulated neurotrophin-coated microspheres were rescued from atrophy.

After four days of MD, neurons labelled with control microspheres in the A (closed-eye) layer of the LGN were 20–30% smaller than control neurons in the adjacent region of the A1 (open-eye) layer (Table 1; Fig. 2a). As previously reported¹⁵, there was no significant difference between the two layers in the average size of LGN neurons projecting to V1 in three animals without lid suture (258 ± 17 versus $268 \pm 29 \mu\text{m}^2$, respectively; $P > 0.3$ by *t*-test or ANOVA). Thus, after as few as four days of MD, neurons in the closed-eye layer of the LGN shrink by approximately 25% relative to neurons in the open-eye layer. This rapid atrophy is consistent with effects of brief MD on geniculocortical terminal arborizations¹⁶.

NT-4, but not BDNF, NT-3 or NGF, prevented this shrinkage. The average somal area for NT-4-treated cells was significantly greater than that of control cells in the closed-eye layer of the LGN, and only slightly smaller than that of control cells in the open-eye layer (Table 1; Fig. 2a). In the closed-eye layer, NT-4 prevented the shift in cell size distribution towards smaller somata that followed MD (Fig. 2b). Neurons in the open-eye layer that contained NT-4-coated microspheres were no larger than control neurons (Table 1; Fig. 2). Thus NT-4 rescued neurons from the effects of MD and did not simply promote growth, as it had no effect on neurons that were not deprived of activity. Further evidence that NT-4 influenced an activity-dependent competitive mechanism comes from the observation that the

shrinkage of LGN neurons in the closed-eye layer was not reversed when NT-4-coated microspheres were injected several weeks after the start of MD (Table 1). Thus there appears to be a critical period for rescue of LGN neurons by NT-4.

BDNF, NGF and NT-3 had no effect upon the average size or size distributions of neurons in either layer of the LGN (Table 1; Fig. 2), although the microspheres carried and maintained the activity of these neurotrophins. Microspheres coated with NT-4, BDNF or NGF all promoted survival of PC12*trkB* cells (Fig. 3). In addition, NT-3-coated microspheres induced phosphorylation of the cAMP response element-binding protein (CREB) in neuroblastoma cells (data not shown).

The ability of NT-4, but not the other TrkB ligand, BDNF, to rescue neurons from effects of MD provides evidence that NT-4 and BDNF have distinct roles in neural development, as has been suggested by recent experiments^{17–19}. The failure of NGF to affect LGN neurons when delivered to their terminals in V1 is in contrast to reports that NGF injected in the ventricles blocks the effects of MD^{8,12}. Although this might be due to species differences, differences in the site and method of delivery are probably more important. NGF infused into the ventricles influences NGF-responsive neurons throughout the brain²⁰. Thus ventricular injection of NGF may alter MD by acting outside the LGN and V1, perhaps affecting modulatory circuits arising from the basal forebrain. Significantly, although NGF and NGF messenger RNA are found within V1 (refs 21, 22), LGN neurons do not express mRNA for the high-affinity receptor for NGF (TrkA)^{23,24}, and do not retrogradely transport NGF when it is injected into V1 (although basal forebrain afferents do²⁵). TrkA receptors have not been demonstrated

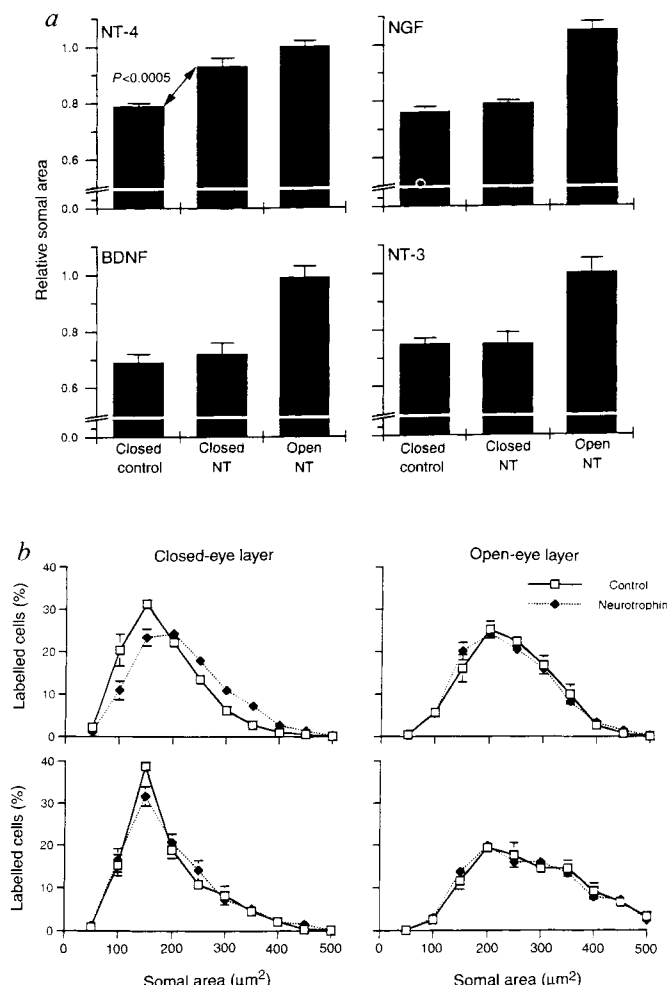


FIG. 2 a, NT-4 rescues neurons from MD-induced atrophy. The effects of MD and neurotrophin treatment were quantified in each animal by comparing the average somal area for control and neurotrophin-treated neurons in the closed-eye layer and neurotrophin-treated neurons in the open-eye layer to the average area of control neurons in the open-eye layer. In every treatment group, control neurons in the closed-eye layer were about 25% smaller than control neurons in the open-eye layer. NT-4, but not the other neurotrophins, eliminated the difference in size between neurons in A and A1. None of the neurotrophins had a significant effect upon neuronal size within the open-eye layer. For each animal, the average somal area of neurons labelled with neurotrophin-coated and control microspheres was determined from camera lucida tracings using NIH Image. The ratios of the average areas of control cells in A, neurotrophin-treated cells in A, and neurotrophin-treated cells in A1 relative to control cells in A1 were calculated for each animal; mean ratios were determined for all of the animals in each treatment group and compared using *t*-tests and ANOVA. b, NT-4 prevents the shift in neuronal soma size caused by MD. Histograms for somal area (mean $\pm$ s.e.m. percentage per bin) are shown for the closed-eye (left) and open-eye (right) layers of the LGN in MD animals injected with microspheres coated with NT-4 (top, $n=8$) or BDNF (bottom, $n=5$). For control neurons in both groups of animals, the distribution in the deprived layer is shifted significantly towards smaller cells relative to the distribution in the non-deprived layer. NT-4 treatment shifted the distribution in the closed-eye layer back towards that seen in the open-eye layer (top left). BDNF treatment had no effect upon the distribution in the deprived layer (bottom left), and neither neurotrophin affected the distribution of cell sizes in the non-deprived layer (right). NGF and NT-3, like BDNF, had no effect upon the size distribution in either layer (data not shown). For each of the four classes of cells (control and treated cells, in A and A1), the percentage of cells in each size bin was determined for each animal. The mean percentage in each size bin was then calculated for all the animals in each treatment group.

on visual cortical neurons or LGN afferents, although mRNA for TrkB is found within thalamic neurons²⁴. TrkB receptors are found in V1 (ref. 26), and NT-4 mRNA is found in cerebral cortex²⁷.

The simplest explanation for our results is that LGN afferents normally compete for NT-4 or a similar endogenous ligand, and the ability of LGN terminals to acquire NT-4 is decreased when their activity is reduced relative to neighbouring fibres. Injecting NT-4-coated microspheres provided an abundant local source

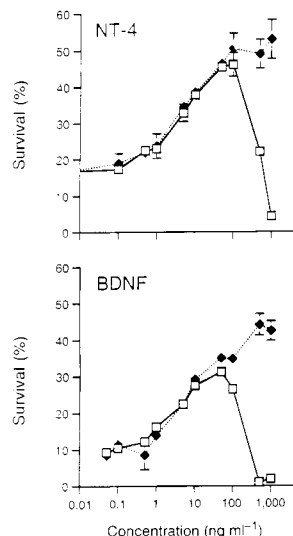


FIG. 3 Fluorescent latex microspheres bind and release active neurotrophin. Neurotrophic activity was quantified as the ability of coated microspheres or free neurotrophin to promote the survival (in serum-free media) of PC12 cells stably transfected with the gene for the TrkB receptor (PC12trkB cells). The percentage of surviving cells relative to parallel cultures treated with 50 ng ml⁻¹ NGF is plotted. NT-4 and BDNF (open squares) promote the survival of PC12trkB cells in a dose-dependent manner. Maximum survival was obtained with NT-4 at 100 ng ml⁻¹ and with BDNF at 500 ng ml⁻¹. Microspheres coated with NT-4 or BDNF (filled diamonds) had a similar ability to rescue PC12trkB cells. NGF-coated microspheres similarly showed potency equivalent to that of free factor (data not shown); NT-3 could not be assayed with this system because the PC12trkB cells do not express the appropriate receptor, TrkC. The concentration of neurotrophin provided by microspheres was calculated given complete binding of neurotrophin to the microspheres (see below). The decreased survival at the highest concentration of NT-4 or BDNF delivered on microspheres is due to a toxic effect on PC12trkB cells of the microspheres themselves at high concentrations; decreases in survival were seen with the same concentration of uncoated microspheres (data not shown). No toxic effects are evident *in vivo* (see Fig. 1 and ref. 30).

METHODS. NGF was purchased from Collaborative Biotechnology; human recombinant NT-4, NT-3 and BDNF were generously provided by Regeneron Pharmaceuticals. Fluorescent latex microspheres (Lumafluor) were ion-exchanged with a mixed-bed ion-exchange resin (BioRad AG 501-X8) and then incubated overnight at 4 °C with one of the neurotrophins. For each preparation of microspheres, 20 µl of microspheres was added to 100 µl of neurotrophin solution (100 ng µl⁻¹). The microspheres were then concentrated by ultracentrifugation (60,000 r.p.m. for 1 h at 4 °C), the supernatant removed, and the microspheres resuspended in 20 µl of sterile water. Preliminary analysis detected only minimal neurotrophic activity in the supernatant, suggesting that the amount of neurotrophin bound to the microspheres was close to the maximum of 500 ng of neurotrophin per µl of microspheres. PC12trkB cells were maintained in DMEM supplemented with 6% fetal calf serum and 6% horse serum (Gibco). For survival assays, cells were plated at high density in serum-free media, and free neurotrophin or labelled microspheres were added. Percentage survival relative to parallel cultures treated with 50 ng ml⁻¹ NGF was assayed 72 h later using a colorimetric cell-proliferation assay (CellTiter 96 AQ, Promega). The data shown reflect the mean ± s.d. for triplicate wells.

TABLE 1 Effects of monocular deprivation and neurotrophin treatment on average somal area of projection neurons in the LGN

Neurotrophin (no. of animals)	Somal area (µm ²)			
	Closed-eye, control	Closed-eye, NT-treated	Open-eye, control	Open-eye, NT-treated
NT-4 (8)	206 ± 9*	242 ± 9†	261 ± 11	259 ± 7‡
BDNF (5)	214 ± 8*	222 ± 8*	310 ± 10	305 ± 5‡
NGF (5)	254 ± 10*	263 ± 4*	333 ± 4	350 ± 7‡
NT-3 (3)	245 ± 18*	242 ± 13*	325 ± 17	323 ± 14‡
NT-4, delivered after MD (3)§	214 ± 18*	233 ± 22*	287 ± 26	308 ± 24‡

For each animal, sections of the LGN in which the binocular region of the A layer and the A1 layer could be clearly discerned were selected. Sections from several animals were combined and coded so that the observer was blinded with respect to which colour microspheres bore the neurotrophin until after the analysis was complete. After a region of labelled neurons were selected for analysis, all of the labelled neurons within a field of approximately 150 × 250 µm were traced using a camera lucida (final magnification ×1030). A new field was then selected and all of the labelled neurons within it were drawn. Labelled neurons were sampled systematically: green and then red cells in A, green and then red cells in the adjacent region of A1. Neurons labelled with a particular colour of microsphere were sampled from the A layer only in sections in which like-labelled neurons could also be sampled from the A1 layer. Neurons were sampled from 4–8 sections, representing approximately 0.25–0.50 mm along the medial–lateral extent of the LGN. An average of 165 ± 7 (mean ± s.e.m.) cells per treatment per layer were drawn for each animal; 16,796 neurons were measured in total. The traced outlines of the neurons were digitized using a flat-bed scanner and the areas were determined using NIH Image.

* $P < 0.05$ versus open-eye control (two-tailed t-test).

† $P < 0.05$ versus closed-eye control (two-tailed t-test).

‡ $P > 0.1$ versus open-eye control (two-tailed t-test).

§ Monocular deprivation initiated P39, NT-4 microspheres injected P65, ferrets killed P69.

of NT-4, thus maintaining trophic support to neurons from the closed-eye layer that would otherwise atrophy. Both NT-4 and BDNF (but not NT-3 or NGF) modulate the formation of ocular dominance columns in the kitten⁷, a process that is also activity-dependent and subject to competitive interactions. Our data demonstrate that NT-4 can rescue neurons from the structural effects of the competitive disadvantage created by decreased activity. Together, these studies provide compelling evidence for a link between activity-dependent, competitive mechanisms and the regulation of neuronal growth and connectivity by neurotrophins. □

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Effects of brain-derived neurotrophic factor on optic axon branching and remodelling *in vivo*

Susana Cohen-Cory & Scott E. Fraser

Division of Biology (139-74), Biological Imaging Center, California Institute of Technology, Pasadena, California 91125, USA

NEUROTROPHINS are thought to be important for the survival and differentiation of vertebrate neurons¹. Roles have been suggested for target-derived neurotrophins, based both on their expression in target tissues at the time of neuron innervation^{2,3}, and on their effects on axonal sprouting⁴⁻⁶. However, direct *in vivo* evidence of their involvement in axon arborization has remained elusive. We have used *in vivo* microscopy to follow individual optic axons over time, and have examined the role of the neurotrophin brain-derived neurotrophic factor (BDNF) in their development. Here we show that injection of BDNF into the optic tectum of live *Xenopus laevis* tadpoles increased the branching and complexity of optic axon terminal arbors. In contrast, injection of specific neutralizing antibodies to BDNF reduced axon arborization and complexity. The onset of these effects was rapid (within 2 hours) and persisted throughout the 24-hour observation period. Other neurotrophins had little or no significant effects. These results demonstrate the involvement of neurotrophins in the dynamic elaboration of axon terminals, and suggest a direct role for target-derived BDNF during synaptic patterning in the developing central nervous system.

The visual system of *Xenopus laevis* tadpoles provides an invaluable *in vivo* system for studying neurotrophin effects on the dynamics of axon arborization and the formation of functional neuronal maps. Individual optic axons, labelled with fluorescent dyes, can be followed in real time as they extend from the retina⁷ and arborize^{8,9} in their target, the contralateral optic tectum. Our previous studies have established BDNF as a relevant molecule during visual-system development in this species. Developing retinal ganglion cells (RGCs) express TrkB, the specific receptor for BDNF^{10,11}, and BDNF is expressed in the optic tectum at the time that the first RGCs project to, and arborize in, the optic tectum¹². Cultured RGCs are dependent on BDNF for their survival and differentiation in *Xenopus*¹² (and other species^{13,14}) during this developmental period, and this correlated dependency suggested that BDNF was an important target-derived trophic factor. Here we directly perturbed the endogenous levels of BDNF, both in culture and *in vivo*, to examine the role of BDNF in axon elaboration and remodelling.

Exposure of dissociated retinal cultures to recombinant BDNF resulted in a significant increase (40%) in average RGC axon length (Fig. 1). In contrast, treatment with function-blocking antibodies against BDNF^{15,16} resulted in a significant decrease (25–30%) in average axon length, whether they were used alone or in combination with exogenous BDNF (Fig. 1), thus suggesting the activity of an endogenous trophic factor. Given the high specificity of the antibody^{15,16}, and our previous findings showing expression of retinal BDNF messenger RNA at this stage¹², these results suggest that the endogenous factor is BDNF itself. An endogenous source of BDNF could account for the significant survival and differentiation of culture *Xenopus* RGCs in the absence of exogenous trophic factors¹². Indeed, RGC survival is diminished by treatment with anti-BDNF (data not shown).

To evaluate the *in vivo* role of target-derived BDNF in the elaboration and remodelling of optic axon terminal arbors, we altered BDNF levels in the optic tectum and examined the effect of these perturbations on the dynamics of individual DiI-labelled RGC axons. Minute amounts of the lipophilic dye DiI were

injected into the retina, labelling 1–2 RGCs and their axons at the stage they project to, and arborize in, the optic tectum. The branching patterns of the DiI-labelled axons were followed in the live animal by confocal microscopy before and after pressure injection into the ventricle and sub-pial space overlying the tectum of BDNF, other neurotrophins (nerve growth factor (NGF), neurotrophin (NT)-3 and NT-4/5), neutralizing antibodies to BDNF, or control solutions (Fig. 2). Three-dimensional reconstructions of each individual DiI-labelled axon followed over time revealed active remodelling of their terminal arbors under all conditions tested. The steady increase in complexity is shown by tracings of representative terminal arbors observed at intervals of 2 hours (Fig. 2c). A greater increase in complexity was observed in the BDNF-treated arbors than in controls, or in those arbors treated with other neurotrophins (Fig. 2c; NGF), whereas little or no increase in complexity was observed following treatment with anti-BDNF.

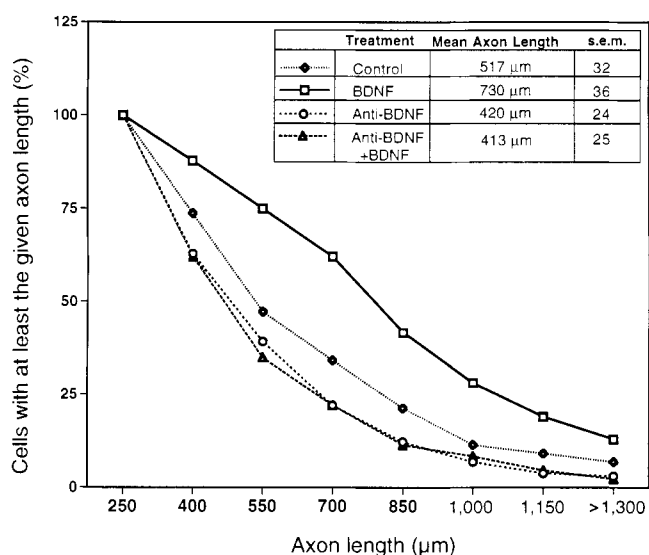


FIG. 1 Exogenous and endogenous BDNF influence RGC axon elaboration in culture. Dissociated retinal cells were grown in culture for 4 days either under control conditions or in the presence of BDNF (20 ng ml⁻¹), anti-BDNF (2 $\mu\text{g ml}^{-1}$), or anti-BDNF + BDNF. Phase-bright neurons with a large- to medium-size cell body (15–20 μm in diameter) and one long axonal process (longer than 150 μm) were scored as RGCs. These cells showed similar morphological characteristics and neurotrophin responsiveness to differentiated RGCs retrogradely labelled from the optic tectum and grown in culture¹². The distribution of RGCs with different axon lengths is plotted as a cumulative histogram. Axon length was measured from a total of 132 RGCs per condition, from duplicate wells per experiment, and from two independent experiments. Mean axon length and standard error of the mean (s.e.m.) are presented in the inset table. Statistical analysis was by one-way ANOVA ($F=24.523$, d.f. = 3,527, $P<0.001$), using a *post-hoc* Fisher protected test. All groups are significantly different from each other, except anti-BDNF versus anti-BDNF + BDNF.

METHODS. Dissociated retinal cells from stage 42/43 *Xenopus laevis* tadpoles were prepared as previously described¹². Human recombinant BDNF (20 ng ml⁻¹; a gift from Genentech) and/or specific BDNF antibodies^{15,16} (2 $\mu\text{g ml}^{-1}$ of purified IgG fraction; a gift from J. Carnahan, Amgen) were added at the time of plating. The antibodies' specificity for BDNF versus other members of the neurotrophin family has been shown by ELISA immunoassay¹⁵, as well as by their ability to block the survival effects of BDNF on cultured cortical and dorsal root ganglion neurons, but not those of NT3 or NT4/5 (ref. 16). Antibody titration studies on cultured RGCs, grown in serum and serum-free conditions, showed that 2 $\mu\text{g ml}^{-1}$ of the purified IgG fraction specifically blocked survival effects of both exogenous BDNF and the endogenous trophic factor (not shown); lower concentrations of the antibody (0.4 $\mu\text{g ml}^{-1}$) were sufficient to block endogenous factor survival effect, but not that of exogenously added BDNF.

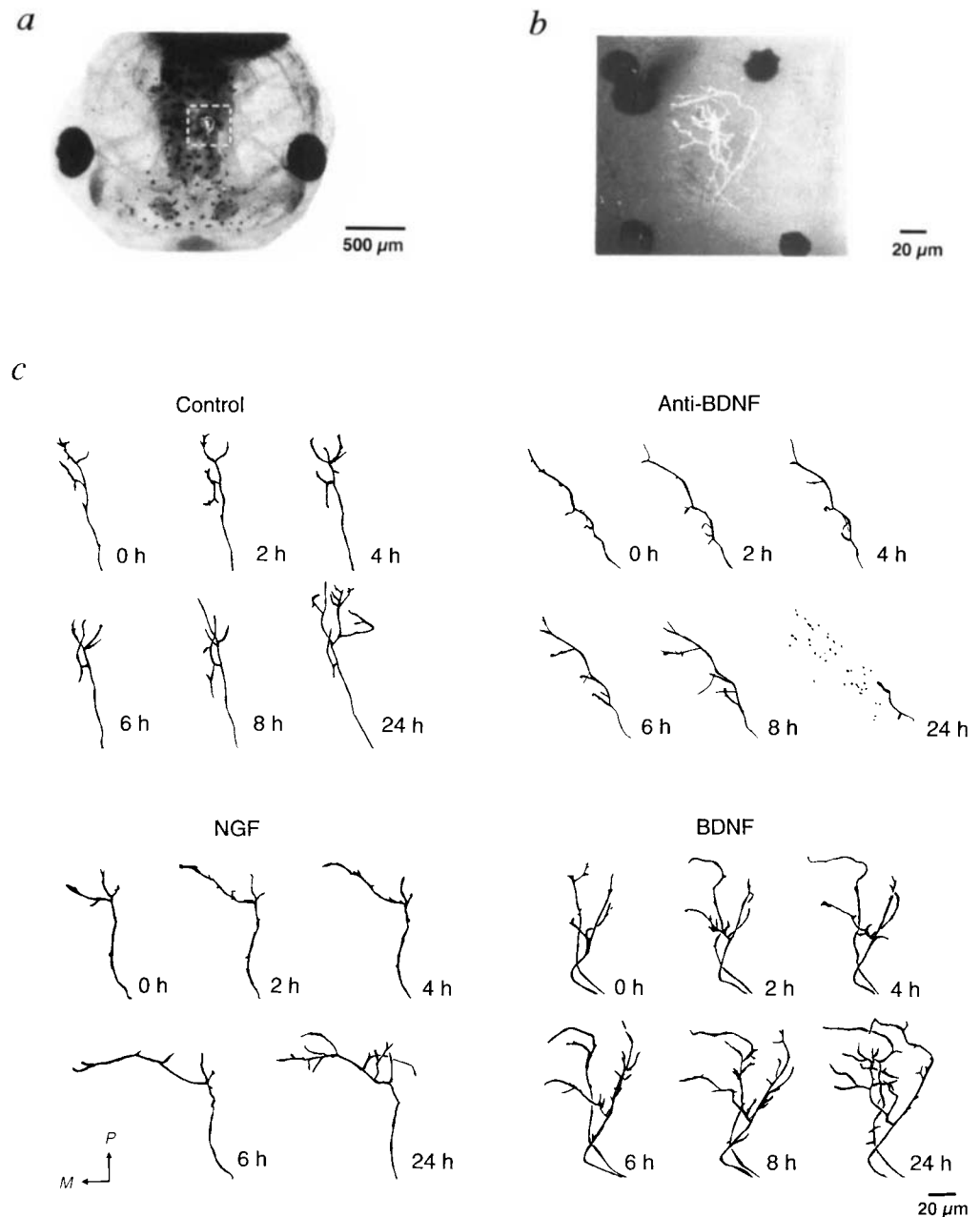
Quantitative analyses of axon morphology indicated that arbor complexity as well as the arborization dynamics were significantly affected by the levels of BDNF in the tectum (Fig. 3a; $n = 18$ per condition). Measurements of arbors imaged every 2 hours for 6–8 hours show that exogenous BDNF significantly increased the addition of individual branches and spikes (branchlets shorter than $5 \mu\text{m}$), as well as the elongation of pre-existing branches (Fig. 3a, top). The number of branches or spikes that retracted or shortened over time was not significantly changed by BDNF (Fig. 3a, bottom). Therefore exogenous

BDNF modulates axon arborization and remodelling mainly by stimulating branch addition and elongation. Reducing the level of endogenous BDNF by injecting function-blocking antibodies to BDNF into the tectum resulted in a complementary effect to that of BDNF: fewer branches were added, although spike increases and branch elongation were normal (Fig. 3a, top). In addition, the antibody elicited a small decrease in branch retraction (Fig. 3a, bottom).

The effects of BDNF and anti-BDNF on branch addition and elongation were rapid, appearing within the first 2 hours of

FIG. 2 Arbor rearrangements visualized over time by confocal microscopy. *a*, Head of a BDNF-treated tadpole, and *b*, higher magnification at the level of the optic tectum showing a fluorescently labelled RGC axonal arbor, here shown in white. *c*, Hand-drawn reconstructions of sample RGC arbors followed over time after tectal injection of: control antibody; anti-BDNF; NGF; or BDNF. Note the extensive branching in the BDNF-treated when compared to control or NGF-treated arbors. Some branches were initially added and retracted in the anti-BDNF-treated arbor, but there was little change in the overall morphology; dramatic degeneration occurred after the first 10 h of treatment. In all frames, posterior is up, medial is left.

METHODS. Stage-41 *Xenopus laevis* tadpoles, reared in modified Rearing solution +0.001% phenylthiocarbamide (to prevent melanocyte pigmentation), were anaesthetized in 1:5,000 Fiquel in Rearing solution, and minute amounts of Dil (Dil(3)-C18, 0.05% solution in 100% ethanol; Molecular Probes, Eugene, OR) were pressure-injected directly into a consistent position in the central retina adjacent to either the nasal or temporal poles. Tadpoles were kept in a fresh Rearing solution, in the dark, for about 16 h (until stage 43/44) to allow complete transfer of the dye to the tip of the axon. Tadpoles having 1–2 clearly distinguishable labelled axonal arbors, each with at least 1–2 simple branches, were selected, and individual optical sections through the full extent of the arbor within the optic tectum were recorded with a Bio-Rad laser scanning confocal microscope. Vehicle solution (0.2–1.0 nl of 50% NiuTwitty solution), recombinant human neurotrophins (200 ng μl^{-1} BDNF, NGF, NT3, or NT4/5; Genentech), anti-BDNF (200 $\mu\text{g ml}^{-1}$), or control IgG (200 $\mu\text{g ml}^{-1}$ rabbit IgG) were pressure-injected directly into the ventricle and sub-pial space overlying the caudalmost tectum. In preliminary experiments, doses of neurotrophins 0.5–5.0 times those presented in this study were tested; these different concentrations showed no significant difference in their effects. Tadpoles were kept in fresh Rearing solution, in the dark, following injection and between observations, and were anaesthetized and imaged every



2 h for 6–8 h. Tadpoles were re-injected 10 h after the first injection, and imaged 24 h after the initial observation. Three-dimensional reconstructions of optical selections of each individual arbor were obtained using the Bio-Rad COMOS software. Immunohistochemical studies confirmed that neurotrophins and function-blocking BDNF antibodies diffuse over the ventricular and tectal surfaces, and penetrate more than $80 \mu\text{m}$ into the cellular layer and into the tectal neuropile where RGC axons arborize (not shown).

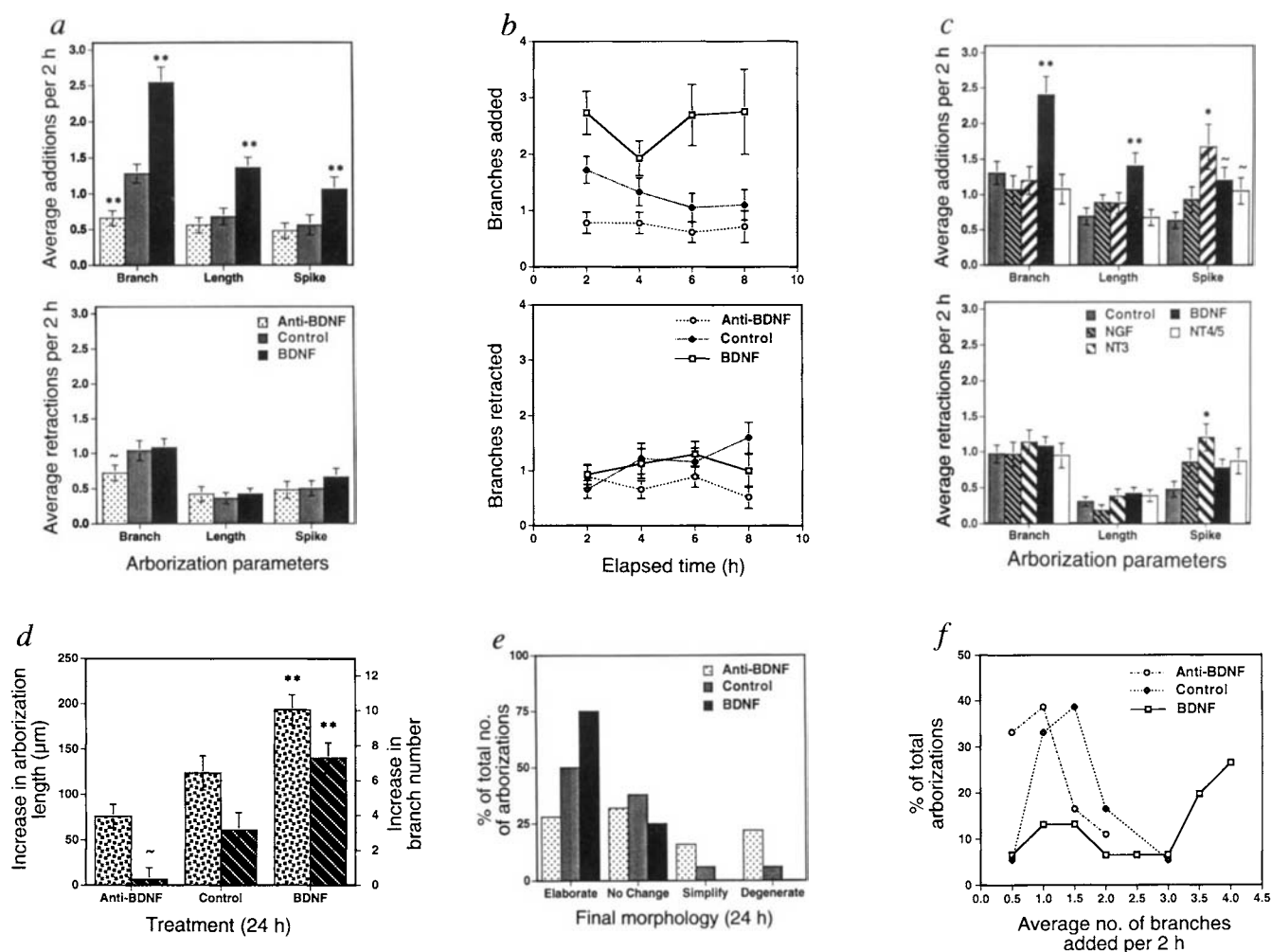


FIG. 3 *a–c*, BDNF modulates the dynamics of RGC axon arborization *in vivo*. The dynamic changes in axon arborization and the rates of branch elaboration in the presence of BDNF or anti-BDNF, or of different neurotrophins, are illustrated by several morphological parameters: the number of branches added, retracted, lengthened or shortened, and the number of spikes added or retracted were quantified and are expressed as average number of additions or retractions per 2-h observation interval. *a*, Average number of additions (top) and retractions (bottom) in control, anti-BDNF, and BDNF arbors ($n=18$ per condition), followed for 6–8 h. *b*, Branch addition (top) or retraction (bottom) in control, BDNF- and anti-BDNF-treated arbors, presented as the average number of changes in each of the 2-h observation intervals, over 8 h. *c*, Average number of additions (top) and retractions (bottom) in control, NGF, NT3, BDNF, and NT4/5 arbors ($n=10$ per condition), followed for 6–8 h. *d*, *e*, The morphology of RGC axonal arbors at 24 h is influenced by tectal BDNF levels. *d*, The effects of BDNF and anti-BDNF on arborization morphology over 24 h were evaluated quantitatively by measuring two parameters: total arbor length (shaded) and branch number (striped). Values are presented as the change from their initial value at the time of treatment. *e*, The relative number of axonal arbors that became more elaborate over time, that did not change significantly in the total observation period, that were simplified by selective pruning of axonal branches, or that degenerated at some point during the 24 h of treatment. *f*, Different responses of two populations of RGC axon arbors to BDNF. The average number of branches added per 2 h for each individual arbor was used as an indicator of responsiveness to BDNF. The histogram shows that a significant fraction of the BDNF-treated arbors branch more actively, but a fraction of those treated with BDNF and anti-BDNF have branching rates similar to those in control arbors. For each test, the mean $\pm$ s.e.m. is shown. Statistical analysis was by one-way ANOVA using *post-hoc* Scheffé-*F* and Fisher tests. *, Significantly different from control, $P \leq 0.01$. **, Significantly different from all other groups, $P \leq 0.01$. ~, Significantly different from control by Fisher PLSD test only, $P \leq 0.03$. In *b* (top), BDNF is significantly different from other groups at all

times; anti-BDNF is significantly different from control at 2, 4 and 6 h ($P \leq 0.01$).

METHODS. Dil labelling, tadpole treatments and arbor imaging were as described for Fig. 2. Morphological parameters from projections of three-dimensional reconstructions of individual arbors were measured to quantify effects on axon elaboration over time. Extensions from the main axon were classified as branches (more than 5 μ m) or spikes (less than 5 μ m). The number of individual branches or spikes gained or lost, and the number of pre-existing branches that changed their length (by at least 20 μ m) from one 2-h observation interval to the next, were scored and counted as '1', regardless of their length change. The data collected for each 2-h observation interval from all arbors followed for 6–8 h are shown as the average number of additions or retractions in each parameter per 2-h time interval. No significant differences in the rates and patterns of axon branching were observed between vehicle and control antibody-treated tadpoles, or between nasal and temporal arbors. For the analysis shown in *c*, data points in control and BDNF include a subset of those arbors analysed in *a*, as well as those followed simultaneously in tadpoles from the same spawnings used to study the effects of other neurotrophins. Data were compiled from at least 4–8 independent experiments for each condition analysed, totalling 17 experiments. For the 24-h end-point analysis, axon length is expressed as the difference in the total length of the main stem, proximal and distal branches at 24 h versus that at 0 h, from all arbors except those that degenerated, and branch number is the difference in the total number of proximal and distal branches between 24 h and 0 h. Values were measured from projections of three-dimensional reconstructions of individual arbors, and are expressed as the mean increase in length or branch number $\pm$ s.e.m. For morphology determination, arbors with a net gain of more than 80% of the initial number of branches were scored as elaborate; those with a net gain of less than 80% were scored as no change; and those with a loss of more than 50% were scored as simplified. Tadpoles in which only labelled membrane particles or degenerating arbors were observed at any point during the 24-h observation period were scored as degenerate.

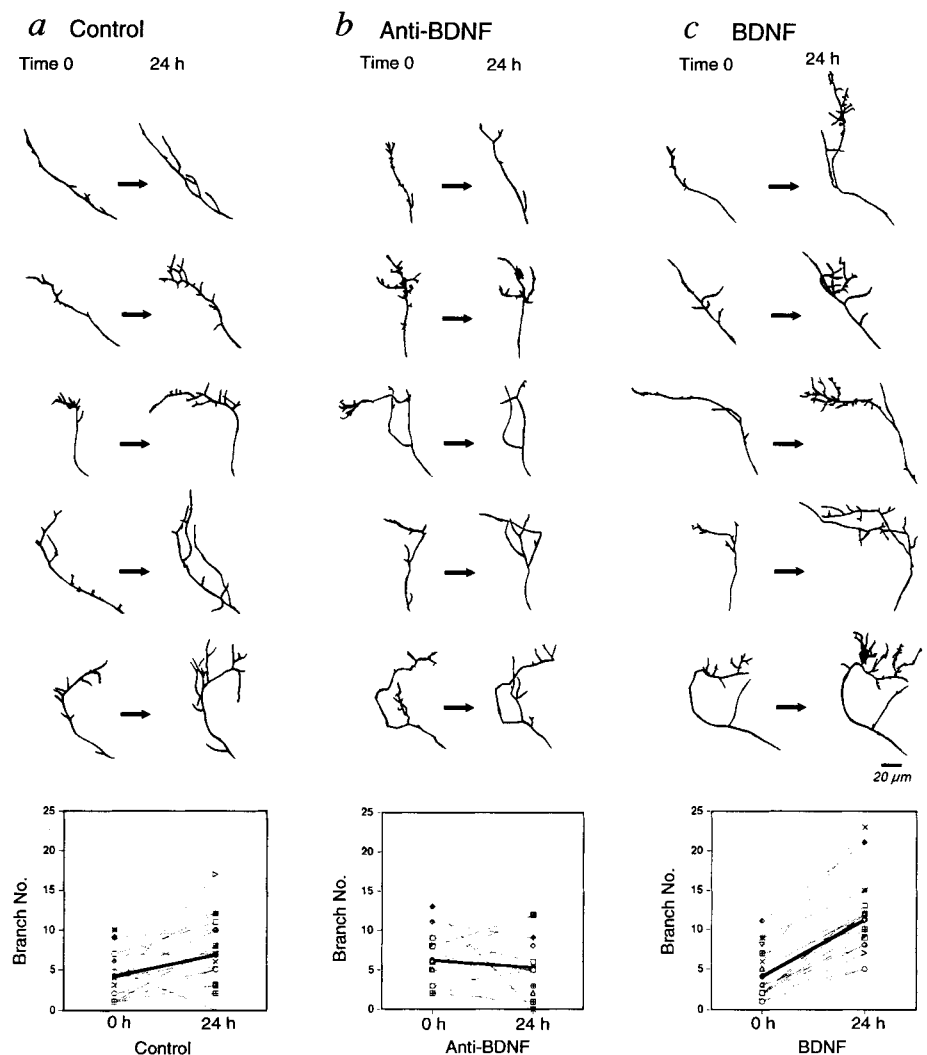
treatment. Analysis of the effects at each 2-hour time point revealed a consistent effect on the rates of branch addition and retraction throughout the 8-hour observation period (Fig. 3b). Furthermore, when scored over a 24-hour interval, the morphology of the arbors was largely consistent with expectations based on the first 2-hour time point. Arbors became morphologically more elaborate over time in the BDNF-treated tadpoles, and simpler in those treated with anti-BDNF in comparison with controls. This is shown by the changes in arbor length and branch number (Fig. 3d), as well as by the proportion of arborizations (Fig. 3e) that became more elaborate in the BDNF-treated tadpoles (75% versus 50% in control) and the fraction that simplified and degenerated in the anti-BDNF-treated tadpoles (40% versus 12% in control). Tracings of sample arbors before, and 24 hours after, treatment further illustrate the effects of BDNF and anti-BDNF on axon arborization, as well as the range of morphologies and responses (Fig. 4, top). Quantitative scatter diagrams of the branch numbers for all arbors analysed show the means and variability of the responses of individual arbors to the treatments (Fig. 4, bottom).

In contrast to BDNF, other members of the neurotrophin family, including NGF, NT3 and NT4/5, had no significant effect on branch addition, elongation or retraction (Fig. 3c). NT3 affected only spike addition and retraction (Fig. 3c), which themselves do not change arbor morphology. NT4/5 only moderately increased spike addition to values similar to those of BDNF (Fig. 3c), whereas NGF had no significant effect on arbor

dynamics (Fig. 3c) or morphology (Fig. 2c). Previous studies have shown that TrkA, the specific receptor for NGF¹⁷, is expressed in the developing vertebrate retina¹⁸, although it is not clear if RGCs express TrkA. In addition, our previous studies showed that TrkC, the receptor for NT3 (ref. 19), is expressed by developing *Xenopus* RGCs¹², suggesting possible roles for this neurotrophin during development. However, of all the neurotrophins tested previously in culture, only BDNF had significant effects on the survival and differentiation of cultured *Xenopus* RGCs¹². Indeed, our present results show that the response of RGCs *in vivo* to increased NT3 tectal levels is different from that of BDNF. Surprisingly, the effects of NT4/5, a neurotrophin that in some systems has been proposed to share the TrkB receptor with BDNF²⁰, differed from those of BDNF both in culture and *in vivo* (results presented here). In culture, NT4/5 increased the survival of only a fraction of RGCs (35% increase in RGC numbers by NT4/5 at doses as high as 100 ng ml⁻¹ versus 75–90% increase by BDNF at doses as low as 0.5 ng ml⁻¹) and had no effect on axon length (unpublished observations). It remains possible that the differences reported here and in other systems²¹ between NT4/5 and BDNF might result from possible receptor binding and/or activation variabilities between the human neurotrophins and the native TrkB ligands. However, the lack of significant effects on axon arborization by neurotrophins other than BDNF suggests that different neurotrophins have different mechanisms of action, and confirms the specificity of the *in vivo* RGC response to BDNF.

FIG. 4 Variability in arbor morphologies 24 h after BDNF or anti-BDNF treatment. Tracings of representative axonal arbors (top) before and 24 h after a, control, b, BDNF or c, anti-BDNF injections, as well as scatter diagrams of branch number for each individual arbor examined (bottom), illustrate the individual variability in arbor morphologies for each condition tested. The bold line in each scatter diagram represents the mean branch number for that treatment.

METHODS. Hand-drawn reconstructions of five representative arbors per condition before (Time 0) and 24 h after initial injection were obtained, as described in the legend to Fig. 2. For scatter diagrams, the number of individual branches at 0-h and at 24-h were evaluated for each arbor per condition ($n=18$ per condition). A score of '0' branches were assigned to those arbors that degenerated at some point during the 24-h observation period. In each diagram, individual arbor data points are represented by different symbols and stippled lines, and the mean number of branches for each condition is represented by filled circles and a bold line.



Not all RGC axonal arbors responded to either BDNF or anti-BDNF. A subset of arbors in tadpoles treated with BDNF or anti-BDNF showed behaviours and rates of branch elaboration similar to those in control-treated tadpoles (Figs 3f and 4). Thus presenting the quantitative data as averages (see Fig. 3a) underrepresents the magnitude of either BDNF or anti-BDNF effects on arborization in the most affected cases. The lack of noticeable effects on a subgroup of RGC axons is consistent with our previous findings that not all RGCs express the *trkB* gene¹². This predicts that a subpopulation of RGCs should be neither responsive to, nor dependent on, BDNF. Indeed, a subpopulation of RGCs survives and differentiates in culture, even in the presence of neutralizing antibodies to BDNF (not shown). Consistent with this prediction of a mixed response, in some cases in which two arbors were followed simultaneously in the same animal, one arbor responded to the BDNF or anti-BDNF treatment but the other did not (in three of four animals). Thus those cases in which the neurotrophin or antibodies had no effect appear to result from a lack of neurotrophin responsiveness by individual RGCs, rather than to failed access of the agents. Consequently, our *in vivo* data indicate that BDNF has a direct effect on axon arborization in most RGCs, probably affecting those expressing TrkB, the specific receptor for BDNF.

The present results show a rapid and significant effect of altering tectal BDNF levels on the dynamics of optic axon terminals *in vivo*. BDNF stimulates the elaboration and extension of axonal branches and spikes; branch elimination continues at the control values. The significant decrease in branch addition in the presence of function-blocking antibodies to BDNF suggests that endogenous tectal BDNF influences the dynamic branching of axonal arbors. Thus these results provide evidence for the involvement of neurotrophins in developmental events other than survival, and suggest a role for BDNF in the interaction of axon terminals with their targets. The rapid BDNF effect on axon arborization and remodelling is consistent with a direct action on optic axons and with possible effects on growth-cone motility, synapse formation, and/or synapse stabilization. Previous studies have shown that axons must dramatically rearrange during retinotectal map formation^{8,9,22,23}, at a time of active transmission of visual information between the retina and optic tectum⁹. Given that neurotrophins have been implicated in modulation of synaptic efficacy²⁴, and that roles have been proposed for coordinated synaptic activity in the formation and refinement of the neuronal maps^{9,25-27}, an attractive hypothesis is that reciprocal interactions between neuronal activity and neurotrophic factors²⁸⁻³⁰ provide a direct means to couple neuronal morphology and function during development of the central nervous system. □

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Defective recycling of synaptic vesicles in synaptotagmin mutants of *Caenorhabditis elegans*

Erik M. Jorgensen^{*†}, Erika Hartweg[†],
Kim Schuske^{*}, Michael L. Nonet[‡], Yishi Jin[†]
& H. Robert Horvitz[†]

^{*}Department of Biology, University of Utah, Salt Lake City, Utah 84112, USA

[†]Howard Hughes Medical Institute, Department of Biology, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, Massachusetts 02139, USA

[‡]Department of Anatomy and Neurobiology, Washington University School of Medicine, St Louis, Missouri 63110, USA

SYNAPTOTAGMIN, an integral membrane protein of the synaptic vesicle^{1,2}, binds calcium and interacts with proteins of the plasma membrane⁴⁻⁶. These observations suggest several possible functions for synaptotagmin in synaptic vesicle dynamics: it could facilitate exocytosis by promoting calcium-dependent fusion³, inhibit exocytosis by preventing fusion⁷, or facilitate endocytosis of synaptic vesicles from the plasma membrane by acting as a receptor for the endocytotic proteins of the clathrin AP2 complex⁸. Here we show that synaptic vesicles are depleted at synaptic terminals in synaptotagmin mutants of the nematode *Caenorhabditis elegans*. This depletion is not caused by a defect in transport or by increased synaptic vesicle release, but rather by a defect in retrieval of synaptic vesicles from the plasma membrane. Thus we propose that, as well as being involved in exocytosis, synaptotagmin functions in vesicular recycling.

In the nematode *Caenorhabditis elegans*, synaptotagmin is encoded by the gene *snt-1* (ref. 9). To characterize the role of synaptotagmin, we examined the synaptic ultrastructure of both wild-type worms and mutants deficient in *snt-1* function, using electron microscopy to determine the number and localization of synaptic vesicles at neuromuscular junctions. We analysed junctions involving three cell types in the ventral nerve cord, the VA, VB and VD motor neurons. Neuromuscular junctions were depleted of synaptic vesicles in *snt-1* mutants compared to wild-type worms (Fig. 1), which had an average of 40 vesicles per synaptic profile, whereas *snt-1(md2665)* mutants had an average of seven vesicles and *snt-1(md290)* mutants had an average of nine vesicles per synaptic profile. Moreover, there was no redistribution of vesicles to the intersynaptic regions: wild-type and *snt-1(md290)* worms both had an average of two vesicles per section between neuromuscular junctions (Fig. 1 legend).

These data suggest that synaptic vesicles may be accumulating in the plasma membrane in *snt-1* mutants. However, alternative explanations are possible. One possible explanation is that the depletion of vesicles at the synapse might be a consequence of the general sickness of the *snt-1* mutants, which have a very slow rate of pharyngeal pumping¹⁰ and, as a result of chronic starvation, are small and have a transparent gut. To determine

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whether starved worms are generally depleted in synaptic vesicles, we sectioned a mutant defective in acetylcholine synthesis, *cha-1(p1152)*¹¹. Like *snt-1* mutants, *cha-1* mutants pump slowly and are starved in appearance. Starvation of both the *snt-1* and *cha-1* mutants is probably caused by the absence of acetylcholine-stimulated pharyngeal pumping¹⁰. The *cha-1* mutant was not greatly depleted in synaptic vesicles, as *cha-1* animals had an average of 32 vesicles per synaptic profile (Fig. 1). A second possible explanation is that the vesicle depletion in *snt-1* mutants might be caused by defects in vesicle transport along the axon. It is believed that immature vesicles are generated from the Golgi and transported to the synapse, and mature synaptic vesicles are formed by an endosomal compartment¹². One possibility is that synaptotagmin is required for the transport of immature vesicles from the Golgi to the synapse. However, *snt-1* mutants did not

accumulate an excess of vesicles in the cell bodies of neurons (Fig. 2a). A third explanation is that *snt-1* mutants might be depleted of vesicles because synaptotagmin is involved in vesicular biogenesis: the Golgi may simply fail to generate synaptic vesicle components. However, synaptic vesicles are present in a *snt-1* strain that also carries a mutation in *unc-104*, a gene that encodes a *C. elegans* kinesin heavy-chain-like molecule¹³. Because of the defect in kinesin, vesicles are not transported along the axon in *unc-104* mutants¹⁴. Vesicles accumulated in the cell bodies of *snt-1 unc-104* double mutants, as visualized using electron microscopy (Fig. 2b). Therefore, vesicles appeared to be generated normally at the cell body in *snt-1* mutants.

To demonstrate more directly that synaptic vesicle components were transported to the synapse in *snt-1* single mutants, we used an expression construct in which the green fluorescent

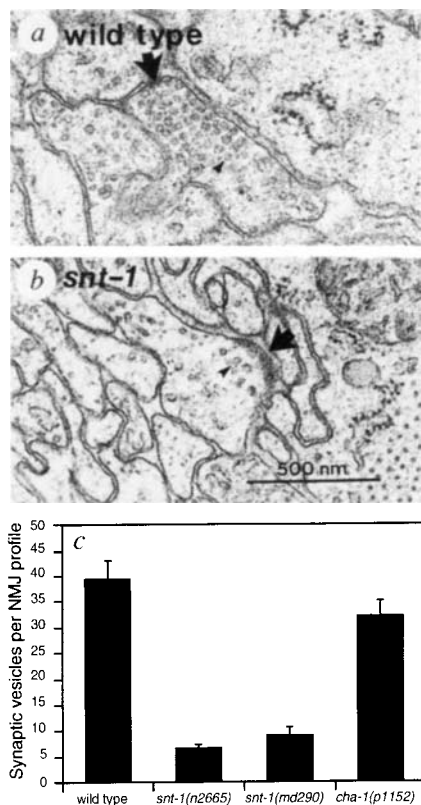
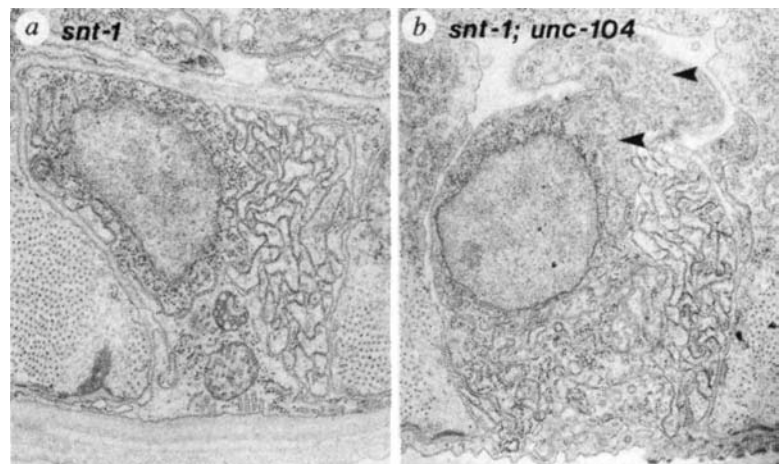


FIG. 1 Synaptic vesicles are depleted at neuromuscular junctions in *snt-1* mutants. **a**, Electron micrograph of a wild-type neuromuscular junction (NMJ). Large arrow, dark thickening of an active zone; arrowhead, one synaptic vesicle. **b**, Electron micrograph of a *snt-1(md290)* neuromuscular junction. **c**, Synaptic vesicles in wild-type (15 NMJs from five worms), *snt-1(n2665)* (15 NMJs from two worms), *snt-1(md290)* (17 NMJs from four worms), and *cha-1(p1152)* worms (17 NMJs from four worms). Synaptic vesicles were counted in a single section that passed through the midpoint of the presynaptic specialization. Error bars represent s.e.m. The *snt-1(md290)* allele should generate a synaptotagmin protein deleted for all but the first 71 amino acids⁹ and thus eliminates part of the transmembrane domain and the two C2 domains. The *snt-1(n2665)* mutation causes a frameshift after Lys 267 and introduces a termination codon 19 codons later, which should generate a truncated protein containing only the first C2 domain (J. A. Crowell and J. B. Rand, personal communication).

METHODS. Worms were cut in 0.8% glutaraldehyde and 0.7% osmium tetroxide in 0.1 M cacodylate (pH 7.4) on ice. After 2 h, they were moved to 2% osmium tetroxide in 0.1 M cacodylate and left at 4 °C overnight. Processing and sectioning were as described previously²⁴. We obtained serial sections from the region of the animal near the anterior reflex of the gonad. We examined the ventral nerve cords for regions containing an active zone or an accumulation of synaptic vesicles and photographed such regions, and counted vesicles at synapses. To quantitate vesicles in intersynaptic regions, we analysed a wild-type motor neuron spanning 50 sections and two active zones, and examined two *snt-1* motor neurons spanning 65 sections containing four active zones each. Sections containing an active zone and two sections on either side of an active zone were eliminated from the data set and vesicles counted in the intervening sections. In wild-type worms there were 2.0 ± 0.3 synaptic vesicles (25–35 nm), 0.9 ± 0.2 large spherical vesicles (~45 nm), and 0.5 ± 0.1 oblong cisternae per section. In *snt-1(md290)* mutants there were 1.7 ± 0.2 synaptic vesicles, 1.2 ± 0.4 large vesicles, and 0.5 ± 0.1 cisternae per section.

FIG. 2 Biogenesis and transport of vesicles are normal in *snt-1* mutants. **a**, *snt-1(md290)* motor neuron cell body in the ventral nerve cord. There was no accumulation of synaptic vesicles in the cell body. Similar results were observed for 13 cell bodies. **b**, *snt-1(md290) unc-104(e1265)* neuronal cell body in the ventral nerve cord. Note accumulation of vesicles in the axonal hillock (arrowheads) of two motor neurons. Similar results were observed for seven cell bodies. To ensure that the vesicles accumulating in the cell bodies contained synaptic vesicle proteins, we demonstrated that two proteins associated with synaptic vesicles are also located in the cell bodies in the *snt-1 unc-104* mutant. First, RAB-3 colocalized with neuromuscular junctions in wild-type animals as visualized with antiRAB-3 antisera. Second, an expression construct in which GFP was fused to the *C. elegans* homologue of the synaptic vesicle protein VAMP (see below) colocalized with synaptic vesicles. Both of these synaptic vesicle markers were found in cell bodies in *snt-1 unc-104* mutants (data not shown).



protein (GFP)¹⁵ was fused to the *C. elegans* homologue of the synaptic vesicle protein VAMP, also called synaptobrevin^{16,17}, and expressed this construct in the DD and VD motor neurons. The DD cell bodies are in the ventral nerve cord; these neurons extend a commissure dorsally and form neuromuscular junctions along a process in the dorsal nerve cord. In wild-type worms the fluorescently tagged VAMP molecule was found in clusters along a process in the dorsal nerve cord (Fig. 3a). The distribution of the fluorescent clusters was consistent in frequency and position with localization at neuromuscular junctions when compared with a reconstructed section of the nerve cord from electron micrographs (data not shown). Although fluorescence was more diffuse in *snt-1* mutants than in wild-type worms, similar levels of fluorescence appeared to be present in the dorsal cords of *snt-1* mutants and wild-type animals (Fig. 3). Additionally, we demonstrated that RAB-3 is transported normally in a *snt-1* mutant. RAB-3 is a *C. elegans* protein related to the vertebrate protein Rab3a, and is expressed by all ventral-cord motor neurons (M.L.N., unpublished observations). As visualized with antiRAB-3 antisera, RAB-3 was localized along the dorsal cord

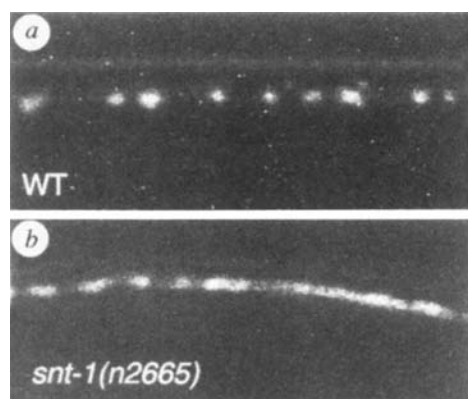


FIG. 3 In *snt-1* mutants, vesicular components are transported to the dorsal cord but localization is diffuse. a, b, Distribution of fluorescently tagged VAMP/synaptobrevin in the dorsal cord of wild-type (a) and *snt-1*(n2665) (b) worms. Both wild-type and mutant worms showed normal transport of the tagged VAMP molecule to the axon terminals. In the *snt-1* mutants the fluorescently tagged VAMP protein appeared diffusely localized along processes instead of at discrete positions along the dorsal cord. Photographs of mutant and wild-type dorsal cords at the level of the anterior reflex of the gonad were mixed and scored in a double-blind test as diffuse or punctuate. *snt-1* was significantly more diffuse: 1 of 10 wild-type and 9 of 10 mutant nerve cords were scored as diffuse (Fisher's exact test, $P = 0.0011$). Similar results were obtained with *snt-1*(md290). These results suggested that VAMP was sequestered in the membrane of the axon and had diffused laterally from the neuromuscular junctions, but we have no direct evidence that VAMP is localized in the plasma membrane.

METHODS. A *C. elegans* VAMP-like gene was cloned using the polymerase chain reaction (PCR). The open reading frame of GFP¹⁵ was inserted at the penultimate codon of a genomic clone of the VAMP gene to yield a fusion to the luminal domain of VAMP (M.L.N., unpublished results). This fusion protein was placed under the control of the *unc-25* promoter, which causes expression in a subset of the neurons which use the neurotransmitter GABA (γ -aminobutyric acid) (RMEs, DDs and VDs; Y.J., unpublished observations). A *lin-15* mutation was used to generate a non-neuronal mutant phenotype to assay successful transformation of co-injected DNA fragments. A *lin-15* rescuing fragment²⁵ and the VAMP-GFP expression construct were co-injected²⁶ into the gonad of *lin-15*(n765ts) mutants. An extrachromosomal array rescuing the *lin-15* phenotype and expressing GFP was stabilized by γ -ray integration into the X chromosome (Y.J., unpublished observations). This fluorescent construct appeared to label synaptic vesicles, as it forms clusters in the ventral and dorsal cords at frequencies approximately equivalent to those of neuromuscular junctions, and because in *unc-104* mutants fluorescence was coincidentally shifted with synaptic vesicles to the cell body (data not shown).

in *snt-1*(md290) animals, as it is in wild-type animals (data not shown). Thus proteins associated with synaptic vesicles were transported normally from the cell body to neuromuscular junctions.

The depletion of synaptic vesicles could be caused by an increased rate of exocytosis, which should increase the efflux of neurotransmitter. To assay acetylcholine release we tested for sensitivity to aldicarb, an inhibitor of acetylcholinesterase¹⁸. Blocking acetylcholinesterase causes toxic levels of acetylcholine to accumulate in the junctional cleft in wild-type worms and causes paralysis; an increase in neurotransmitter release in the mutant should cause hypersensitivity to this drug. We found that *snt-1* adults can tolerate fivefold higher concentrations of aldicarb than can wild-type worms before being paralysed (Fig. 4). These results suggest that acetylcholine release is decreased at neuromuscular junctions in *snt-1* mutants. In support of this conclusion, electrophysiological recordings from pharyngeal muscles of intact *C. elegans* indicated a decrease in neurotransmission from a class of glutamatergic motor neurons in *snt-1* mutants¹⁹ (J. A. Dent, M. W. Davis and L. Avery, personal communication). In addition, acetylcholine accumulates at abnormally high levels in *snt-1* mutants⁹. These observations are inconsistent with there being an increased rate of exocytosis in *snt-1* mutants and suggest that these mutants are deficient in neurotransmitter release. We propose that a null mutation in synaptotagmin leads to a decrease in transmitter release by blocking the regeneration of synaptic vesicles and depleting the synaptic termini of vesicles.

Synaptic vesicle components are recovered from the plasma membrane by endocytosis^{20,21}. The simplest interpretation of our data is that synaptotagmin is required for endocytosis of synaptic vesicles. This possibility is supported by the finding that rat neuronal synaptotagmin I binds clathrin AP-2 (ref. 8), a complex of proteins required for the formation of coated pits. Moreover, other synaptotagmin genes expressed in non-neuronal tissues also bind clathrin AP-2 (ref. 22). These data indicate that synaptotagmin might function as a plasma membrane receptor for the endocytotic machinery in many tissues. After endocytosis, vesicular components are sorted and reformed into vesicles from an endosomal compartment¹². It is also possible that synaptotagmin functions in the regeneration of synaptic vesicles from

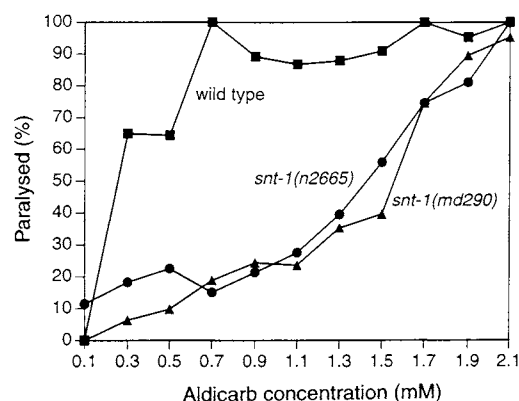


FIG. 4 Synaptotagmin mutants are resistant to an inhibitor of acetylcholinesterase.

METHODS. We placed 20 *snt-1*(n2665), *snt-1*(md290) or wild-type worms on agar plates seeded with bacteria and containing aldicarb, an inhibitor of acetylcholinesterase. Chronic application of aldicarb does not severely affect the generation time of *snt-1* mutants⁹. To assay directly the effect of aldicarb at neuromuscular junctions, we assayed acute paralysis in adult animals. Worms were assayed for movement and pharyngeal pumping 8 h after exposure to the drug. Paralysis is defined as lacking both movement of the body and pumping of the pharynx.

the endosome rather than in membrane retrieval at the plasma membrane.

Our conclusion that synaptotagmin has a role in vesicular recycling does not preclude synaptotagmin from also having a role in exocytosis; we simply find that the primary defect seen in the *C. elegans* mutant is at the step of vesicular recycling. We propose that synaptotagmin functions to regulate both exocytosis and the recycling of synaptic vesicles^{2,3}. □

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Role of SUPERMAN in maintaining *Arabidopsis* floral whorl boundaries

Hajime Sakai, Leonard J. Medrano & Elliot M. Meyerowitz

Division of Biology, 156-29, California Institute of Technology, Pasadena, California 91125, USA

THE *Arabidopsis* gene *SUPERMAN* (*SUP*) is necessary for the proper spatial development of reproductive floral tissues^{1–3}. Recessive mutations cause extra stamens to form interior to the normal third whorl stamens, at the expense of fourth whorl carpel development^{1–3}. The mutant phenotype is associated with the ectopic expression of the B function genes, *AP3* and *PI*, in the altered floral region, closer to the centre of the flower than in the

wild type³, and *ap3 sup* and *pi sup* double mutants exhibit a phenotype similar to *ap3* and *pi* single mutants. These findings led to *SUP* being interpreted as an upstream negative regulator of the B function organ-identity genes, acting in the fourth whorl^{2,3}, to establish a boundary between stamen and carpel whorls. Here we show, using molecular cloning and analysis, that it is expressed in the third whorl and acts to maintain this boundary in developing flowers. The putative *SUPERMAN* protein contains one zinc-finger and a region resembling a basic leucine zipper motif, suggesting a function in transcriptional regulation.

The phenotypes of wild-type and *SUP* mutant flowers are shown in Fig. 1a and b. The *SUP* gene was mapped to the middle of chromosome 3 between the restriction-fragment length polymorphism (RFLP) markers λ At105 and KG-23, in close vicinity to λ At255, with a distance of 1–2 cM³. We thus used λ At255 (ref. 4) as the starting point for a chromosome walk towards the *SUP* locus. We isolated 600 kb of contiguous genomic DNA by screening yeast artificial chromosome (YAC)

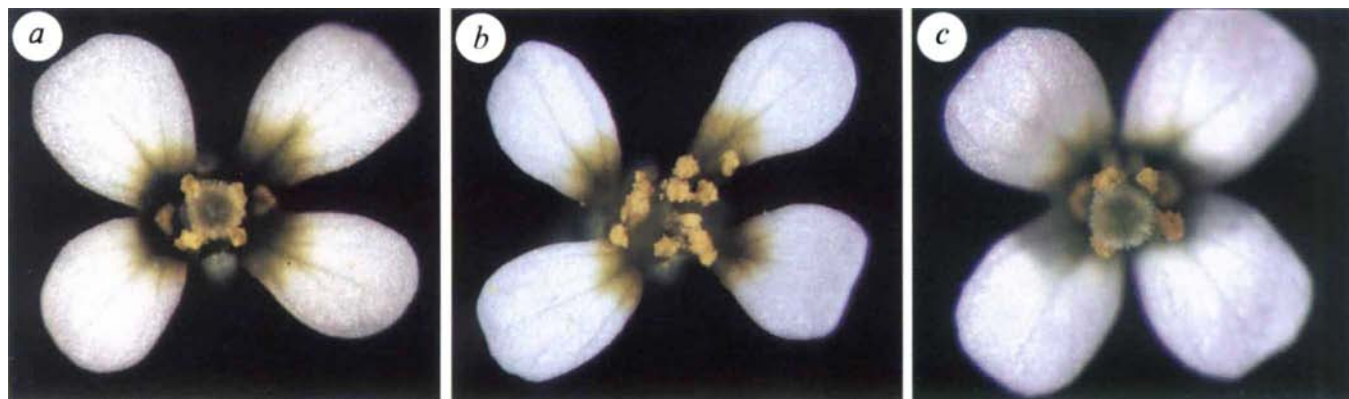
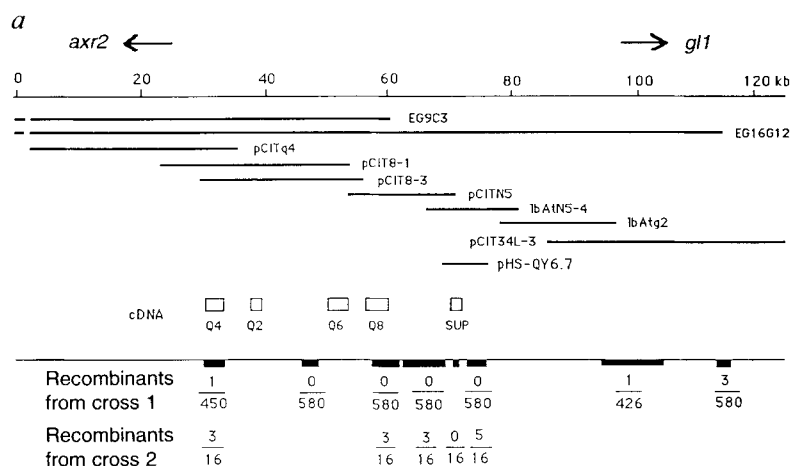


FIG. 1 a, Wild-type flower; b, *superman-1* mutant; and c, *superman-1* with transgenic *SUP* gene. a, Wild-type flower consists of four whorls, occupied by four sepals, four petals, six stamens and two fused carpels from the outermost first whorl to the innermost fourth whorl. b, The flower of *superman* forms extra stamens interior to the third whorl stamens and a reduced carpeloid organ in the centre of the flower. c, *superman-1* plants carrying the transgenic wild-type *SUP* gene produce flowers indistinguishable from wild type.

METHODS. For the complementation test the genomic clone pHS-QY6.7 in a pCGN1547 vector (Calgene), which contains the *SUP* coding region with 5.3 kb of upstream and 0.7 kb of downstream sequences, was introduced into the *Agrobacterium tumefaciens* strain ASE (Monsanto). This strain was used to infect wild-type No-0 plants by the vacuum

infiltration method²². Two independently isolated kanamycin-resistant transformants were then crossed to *sup-1 gl1-1* strains. The *gl1-1* recessive mutation, which is approximately 12 cM away from *sup*^{2,3}, was used to screen potential *sup-1* homozygous plants in the F₂ progeny. All 28 Km^R *gl1* plants from these crosses produced flowers indistinguishable from wild type. The homozygous state of *sup-1* in these *gl1* plants was confirmed by amplification of the *sup* locus by the polymerase chain reaction (PCR) using one primer from the 5' region of *SUP* and another from 1.2 kb downstream of *SUP*, which was not present in pHS-QY6.7, and followed by genotyping through cleaving DNA with *Nco*I, which has a cleavage site in the wild-type sequence but not in the *sup-1* mutant allele used.

FIG. 2 *SUP* gene region and sequences. a, Map of genomic clones and cDNA clones. b, The cDNA and deduced amino acid sequence (single-letter code) of the *SUPERMAN* gene (Ler wild type). The intron of 106 nucleotides, which is identified by comparison with the genomic sequence, is shown in lower-case letters. Numbers to the right indicate the position relative to the putative translational start site. The zinc-finger domain and the putative coiled-coil region are underlined. Two overlapping leucine zipper-like repeats are indicated with a and b. The basic residues that may serve as a nuclear localization sequence are shown in bold letters. The existence of a stop codon upstream of a putative translational start codon, and in frame with the open reading frame, in the cDNA clone indicates that the cDNA clone contains the full coding region. The mutations in *sup-1* and *sup-4* cause a change of Trp 22 (TGG) to a stop (TGA); the mutation in *sup-2* causes a change of Trp 22 to a different stop codon (TAG). The *sup-3* allele causes a missense mutation at Gly 63 (GGT) to Asp (GAT). c, Comparison of the *SUP* zinc-finger with other zinc-finger sequences, which are derived from the following sources: Petepf11 and Petepf12 (EPF1 from *Petunia*)²³; Petepf5a1 and Petepf5a2 (EPF2-5a from *Petunia*)²⁴; Petepf71 and Petepf72 (EPF2-7 from *Petunia*)²⁴; Triwzf1 (WZF1 from wheat, accession no. S39045); Drozf2 (zfh-2 from *Drosophila*, P28167); Krüppel (Kr from *Drosophila*)⁶; Buftf1ia (TFIIA from American toad, P34694); Homznf75 (ZNF75 from human, S67970); and Sacadr1 (ADR1 from yeast, P07248). The proteins with zinc-fingers most closely related to that of *SUP* are from *Petunia*^{23,24}. The amino acid sequence adjacent to the first histidine residue of the zinc-fingers of these proteins is identical to the corresponding region in *SUP*: QALGG. This region includes some of the residues of Cys 2 His 2 type zinc-fingers that have previously been shown to interact with DNA²⁵. The mutational lesion in the *sup-3* allele is a change of the first glycine in the QALGG sequence to aspartate. Because the phenotype of *sup-3* is the same as that caused by the other nonsense mutations, this residue seems to be essential for *SUP* function. METHODS. The cosmid and lambda clones were isolated by screening pCIT30-borne²⁶ and λGEM11(Promega)-borne genomic libraries with DNA fragments from YAC EG16G12 (ref. 27). These subclones were used to screen a flower-specific cDNA library in the λZAPII vector (Stratagene)²⁸, and to map recombinations from cross 1, *sup-1* (Ler) × *gl1-1* (Co), and cross 2, *sup-1 gl1-1* × *axr2-1*. The numbers of cross 1 show the fraction of recombinations in chromosomes from a total of 290 F₂ progeny. In cross 2, 16 recombinants between the *sup* and *axr2* loci were obtained from a total of 2223 F₂ progeny and analysed for their recombination break points. The observed genetic order of three loci is *axr2-sup-gl1*. The originally isolated cDNA clone was the only one found among 2 × 10⁶ clones derived from young floral apices. Similar experiments with probes specific for the floral organ-identity genes *AP3*, *Pl* and *AG* showed that their cognate cDNA clones are present at a frequency of 2 × 10⁻⁴ to 4 × 10⁻⁴ in the same cDNA library. This indicates that *SUP* RNA is several-hundred-fold less abundant than the transcripts of these other genes in young flowers. The length of the cDNA is consistent with the transcript size detected on the poly(A)⁺ RNA blots (approximately 1.5 kb). To obtain the mutant sequence, lambda genomic libraries in the GEM11 vector (Promega) were constructed from *Sau*3A-partially digested DNA of *sup-1* and *sup-2* plants. The clones containing the *SUP* region were obtained by screening the libraries with the *SUP* cDNA pHS-QY; 5.6 kb and 1.2 kb *Eco*RI fragments of these clones were subcloned into pBluescriptIIISK⁺ (Stratagene) and double-strand sequenced using a Sequenase version 2.0 kit (USB). For *sup-3* and *sup-4* alleles the *SUP* genomic region was amplified by PCR using a pair of primers, 5'-GAGACAGACAGACATAGA-3' and 5'-TGTAACCTCGAATCC-3'. The products from two independent PCR reactions sequenced using nested primers. The mutations of *sup-*



b

		ATCTCTCTCTCTCTCTTAAGA	-181
GACGACAGACATAGATATATCTTAGATT	TTC	CCGGGAAAAAAGlgagtcattctt	-122
tcaagatcttacaacactgttggtttatataa	cgtgta	accacaacatctactagtact	-61
atcttaattatctttlacaattgtttttatc	tagc	TAGGCCAAAGGAAGCGTTGCACAT	-1
ATGGAGAGATCAAAACAGCATGTGGTGA	AACAGCTT	CATGCGCGTGCAAGAACCTCA	60
M E R S N S I E L R N S F Y G R A R T S			20
CCATGGAGCTATGGAGATTATGATAATT	GCCAACAGGATCATGATTATCTT	CATAGGTTT	120
P W S Y G D Y D N C Q Q D H D Y L L G F			40
^ ^{sup-1,-2,-4}			
CTATGGCCACCAAGATCCTCACTTGCAG	CTTCTGCAAAGGGAATTCAGATCGG	CTCAA	180
S W P P R S Y T C S F C K R E F R S A O			60
GCACCTGGTGCCACATGAATGTTTACAG	AGAGACAGACGAGACTCAGATTACAAC	AG	240
A L G G H M N V H R R K D R A R L R L Q Q			80
^ ^{sup-3}			
TCTCCATCATCATCTTCAACACCCTTCT	CCTCTACCTTACCCTAATTA	TACTCTTACTCA	300
S P S S S T P S P F Y P N P N Y S Y S			100
ACCATGGCAAACCTCTCCTCCTCATCAT	TCTCCTTCAACCTAATTTCCAACCC	TTTCT	360
T M A N S P P P H H S P L T L F P T L S			120
CCTCCATCTCACCAAGATATAGGCGGTT	GTGATCCGTTCTTGAGCCCCAACG	CAAAA	420
P P S S P R Y R A G L I R S T S P K S K			140
CATACACCAGAAAAACGTTGTAAGACTA	AGAAATCATCTCTTTTACTGGAGGCT	GAGAG	480
H T P E N A C K T K K S S L L V E A G E			160
GCTACAAGTTTACCAGTAAAGATGCTT	GCAGAGTCTGAGGAATGATGAAATCAT	CAGC	540
A T T R F T S K D A C K L B N D E I J S			180
a. a. a.			
TTGGAGCTTTGAGATTGGTTTGATTAN	CAEACAGATCTGGATCTAGAACTCCG	T	600
L E L E I G L T N E S E Q D L D L E L R			200
b. a. b. b. b.			
TTGGGTTTCGCTTAATTAGATGGTAATA	AACTTTATCCATAAAGGATTCGAAGT	TCACAAT	660
L G F A * *			
CTTGAAGATATAGTGCTTCTCTCAAGT	TAACTAGTTTCATCCATGAAATTTCT	TAAG	720
CTGCTATTAGTAGAAGCGTTATAGTTG	ATTATGATTATAGTATAGTAAAGCTA	TAAAGCTTG	780
CGTTAGATGTTATATAATATGTAATGCT	GTTTGTACC GTTTTATGATGACAAAT	TTGTATACA	840
TTTTGTCTCCTTAATTATTTGTTATCTC			

[illegible]

sup-4 were further confirmed by DNA gel blot analysis, which showed the altered *Nco*I restriction site caused by these mutations.

libraries (data not shown). The RFLP mapping of meiotic recombination breakpoints between *SUP* and surrounding DNA allowed us to localize the *SUP* gene to a single YAC clone, EG16G12. Further subcloning of this YAC region and isolation of recombinants between *sup* and a nearby locus, *axr2* (refs 2, 5), narrowed down the *sup* gene to a region of 15 kb that hybridized to only one complementary DNA clone (Fig. 2a). We confirmed that this clone represented the *SUP* gene by sequencing the cognate DNAs from wild-type plants and from four independent *sup* mutants (Fig. 2b). In addition, full complementation of the *sup* mutant phenotype was obtained by introduction of a genomic DNA fragment containing the gene and surrounding sequences to mutant plants (Fig. 1c).

The open reading frame of the *SUP* gene encodes a protein of 204 amino acids. Sequence comparison revealed that one region of the predicted SUP protein is similar to Cys 2 His 2 type zinc-fingers. This motif is present in several transcription factors, such as *Drosophila* Krüppel and *Xenopus* TFIIIA^{6,7} (Fig. 2c). SUP contains only one finger motif, but most other known

proteins of the Cys 2 His 2 type contain more than one finger. In this respect, SUP resembles the GAGA factor of *Drosophila*, a DNA-binding protein that controls homeotic and other genes and that also contains one Cys 2 His 2 type zinc-finger⁸.

Carboxy-terminal to the SUP zinc-finger is a region rich in serine (13 of 45 residues) and proline (14 of 45). In *Drosophila*, similar proline-rich sequences are thought to act as transcriptional activation or repression regions^{9,10}. Further towards the C terminus of the protein is a cluster of basic residues that might serve as a plant nuclear localization signal¹¹, followed by a stretch that could form a coiled-coil structure. This stretch contains two overlapping leucine zipper-like motifs, which could potentially form two staggered hydrophobic regions, as has been proposed for a heterodimerization domain of *Saccharomyces* MATa1 (ref. 12). All of the protein motifs suggest that SUP acts as a transcription factor.

SUP RNA is detected only in flowers by RNA blot analysis (data not shown). To determine which floral cells accumulate SUP RNA, *in situ* hybridization was performed on sections of

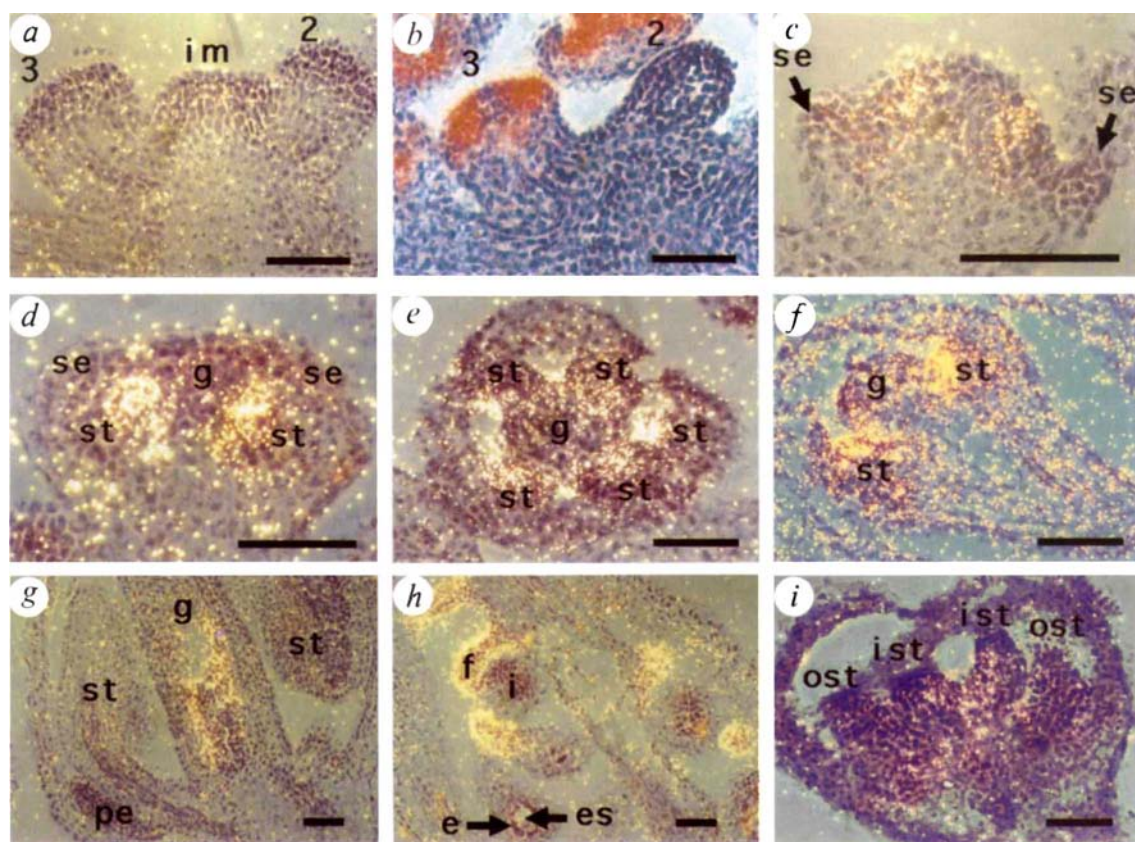
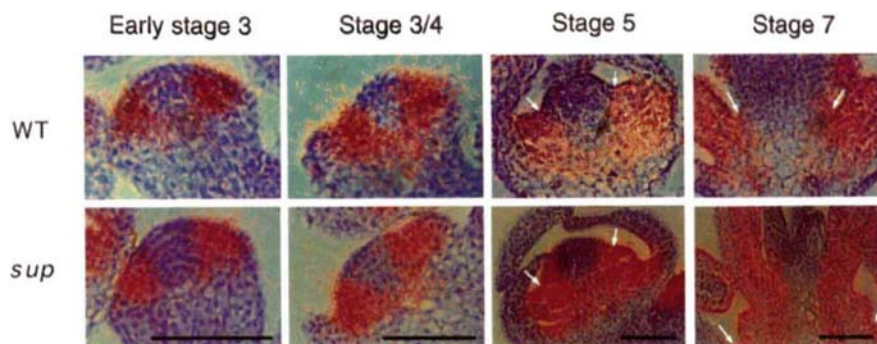


FIG. 3 Expression patterns of *SUP* in wild-type (Ler) and p35S-AP3 mutant flowers. a, c–i, *In situ* hybridization with *SUP* antisense probe; b, with AP3 antisense probe. Each section was photographed with brightfield–darkfield double exposure (using a yellow filter for *SUP* and a red filter for AP3 during darkfield exposure). a, b, Stage 2 and early stage 3 wild-type flowers (stages according to ref. 13); im, inflorescence meristem. c, Wild-type late stage 3 flower; se, sepal primordium. The number of epidermal cells that do not express *SUP* were counted across the central region of a number of late stage 3/early stage 4 flowers; 6.7 ± 0.7 cells (mean $\pm$ s.d., 7 flowers counted) are in the non-expressing core. The core of epidermal cells that do not express AP3 at this stage is the same diameter (see Fig. 4). d, Wild-type stage 4 flower; st, stamen; g, gynoecium primordia. e, Wild-type stage 6 flower (sectioned transversely). f, Wild-type stage 6 flower. g, Wild-type stage 9 flower; pe, petal primordium. h, Carpels of a wild-type stage 15 flower; f, funiculus; i, integument; e, endothelium; es, embryo sac. i, A p35S-AP3 stage 7 flower; in this stage *SUP* expression is detected in the

inner side of the inner whorl stamen primordia (ist), but the expression on the outer stamen primordia (ost) has declined. Scale bars, 50 μ m. METHODS. To synthesize the *SUP* antisense probe, which did not include the zinc-finger region, the clone pHSup3 in the pCRII vector containing the PCR-amplified DNA of the *SUP* genomic region was linearized by *MscI* digestion and *in vitro* transcribed in the presence of [³⁵S]UTP with T7 RNA polymerase. Further *in situ* hybridization experiments were performed as described previously¹⁴, with the following modifications: (1) tissue was fixed for 2 h; (2) sections were incubated in $2 \times$ SSC for 15 min at 70 °C after dewaxing and hydration through an ethanol series; (3) sections were postfixed in 4% paraformaldehyde in PBS for 20 min after the proteinase K treatment; (4) sections were hybridized with probe at 45 °C; and (5) they were exposed with emulsion for 8–9 weeks. Hybridizations with a sense probe showed no specific hybridization pattern and few background grains. AP3 *in situ* hybridization was performed as described previously¹⁵.

FIG. 4 Expression patterns of *AP3* in wild type and the *sup-1* mutant at four different stages of flower development. The boundary between the outermost stamen primordia and the interior primordia (of stamens in the *sup* mutants, of carpels in wild type) is shown by arrows. For comparison, the pictures of wild type and the *superman* mutant were taken at the same magnification in each stage. Scale bars, 50 μ m. No consistent difference in *AP3* expression was observed between *sup* and wild-type flowers in stages 3 and 4. The epidermal cell number counted across the central region where *AP3* is not expressed in sections of stage 3–4 flowers is 7.0 ± 0.8 cells (mean $\pm$ s.d.) in *sup* mutant flowers (7 counted), and 6.9 ± 0.8 (14 flowers counted) in wild type. In *sup* mutants the central region shows no increase in cell number as development proceeds, but in wild type this region enlarges throughout flower development.



METHODS. The longitudinal sections of the wild-type and *sup-1* flowers were probed with the 35 S-labelled antisense *AP3* RNA as described previously¹⁵. The sections were exposed with emulsion for approximately 2 weeks.

developing wild-type flowers, using a *SUP* antisense probe. The earliest *SUP* expression detected was in late stage-3 flower primordia (when the sepal primordia are first becoming distinct from the floral meristem; stages as in ref. 13). Thus *SUP* RNA is first detected after the earliest detectable expression of the B and C function organ-identity genes (Fig. 3a–c)^{14–16}. The early domain of *SUP* expression is adjacent to the boundary between the third and fourth whorls, coincident with a subset of the domain of *AP3* expression (Fig. 3c). In later floral stages *SUP* RNA is found in the inner (adaxial) portion of the developing stamen primordia (Fig. 3d–f). The domain of this stage of *SUP* expression corresponds to the inner part of whorl 3. By stage 8, when stamen and carpel primordia become clearly separate, *SUP* RNA is found in the part of developing stamens adjacent to the carpel primordia. Later, in stage 9, *SUP* RNA becomes detectable in the developing ovary, first in the inner surface of the carpels, and later in the stalk (funiculus) of each ovule (Fig. 3g). During ovule development *SUP* RNA continues to be present in the funiculi, but not in the embryo sac, the nucellus or the integuments (Fig. 3h).

That the initial detectable appearance of *SUP* RNA follows that of *AP3* RNA implies that the negative action of *SUP* on *AP3* expression occurs after the establishment of the normal early domain of *AP3* RNA expression. To verify this, *in situ* hybridization was done on sections of late stage-3 *sup-1* mutant floral primordia using *AP3* antisense probes. At this stage, which is approximately the same time as the onset of detectable *SUP* expression, the *AP3* expression domain appears normal. Only later does *AP3* RNA appear closer to the floral centre than in the wild type; *AP3* RNA gradually extends into the central region during stages 4–8 (Fig. 4). A demonstration that *SUP* activation is downstream of *AP3* is that ectopic expression of *SUP* is detected in the flowers of p35S-*AP3*, which is constitutively expressing *AP3* and has persistent *PI* and *AP3* expression in the fourth whorl¹⁷. The phenotypic result is the formation of stamens interior to the third whorl stamens, similar to the *sup* mutant phenotype. In stages 7 and 8, when the inner stamen primordia are being formed, *SUP* is expressed on their inner side (Fig. 3i). The ectopic expression of *SUP* in p35S-*AP3* transformants, and the detectable expression of *AP3* earlier than *SUP*, indicate that *SUP* is regulated by *AP3*. This is confirmed by the observation that *SUP* RNA is much reduced or absent in *ap3* mutant flowers, as shown by *in situ* hybridization (data not shown). The results also demonstrate that *SUP* does not act directly in the fourth whorl to establish the prepattern of *AP3* expression, as was previously proposed. Thus the earlier model in which *SUP* regulates the initial pattern of *AP3* expression by acting in the fourth whorl is incorrect.

Two alternative explanations fit both the earlier genetic and the present molecular data. One is that the function of *SUP* is

to form a boundary between third and fourth whorl cells that acts to prevent the spreading of *AP3* activity from the third to the fourth whorl³. Another explanation is that *SUP* is involved in cell division in developing flowers. If *SUP* normally acts to repress cell division in those cells where it is expressed, eliminating *SUP* activity would cause proliferation of cells in the inner part of the third whorl, with consequent formation of extra stamens. To explain the reduction of the fourth whorl carpels that is also seen in *sup* mutants, we must consider that excess cell division in the third whorl (or some other non-autonomous action of *SUP*) results in a repression of cell division in the fourth whorl. The repressive activity of *SUP* would be antagonistic to the demonstrated activity of *AP* and *PI* in promoting cell division in the region^{15,18,19}. The epistasis of *ap3* and *pi* in double-mutant combinations with *sup* would thus reflect that the B function genes are positive regulators of *SUP*, and/or that *AP3/PI* and *SUP* act antagonistically. *SUP* has recently been found to have a function in developing ovules as well as in developing flowers²⁰, and *sup* ovules show overgrowth of the integuments. Such a phenotype also indicates a role of *SUP* in cellular proliferation. Because *SUP* is detectably expressed not in the integuments but in the funiculus, it is possible that *SUP* acts cell non-autonomously in ovule development.

In either of the new models, *SUP* acts to maintain a boundary between adjacent groups of cells after their difference (as reflected in their differential expression of *AP3*) has already been established. Boundary-maintenance functions during development also exist in animal systems. In *Drosophila*, for example, compartment-specific cellular proliferation and cell patterning are maintained by a set of genes whose regulation is mediated by gene products secreted across the compartment boundary²¹. Whether proteins similar to those of the *wingless* and *hedgehog* genes are involved in maintenance of the third-whorl/fourth-whorl boundary in *Arabidopsis* flowers remains unknown.

Note added in proof: *Arabidopsis* genes encoding similar zinc fingers and C-terminal regions to *SUP* were recently reported²⁹. □

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Rescue of early embryonic lethality in *mdm2*-deficient mice by deletion of *p53*

Roberto Montes de Oca Luna, Daniel S. Wagner* & Guillermina Lozano†

Department of Molecular Genetics, and * Department of Biochemistry and Molecular Biology, University of Texas, M.D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, Texas 77030, USA

THE gene *p53* encodes a transcriptional activator^{1,2} of genes involved in growth arrest^{3,4}, DNA repair⁵ and apoptosis^{6–8}. Loss of *p53* function contributes to tumour development *in vivo*^{9–11}. The transcriptional activation function of *p53* is inactivated by interaction with the *mdm2* gene product^{12–14}. Amplification of *mdm2* has been observed in 36% of human sarcomas, indicating that it may represent an alternative mechanism of preventing *p53* function in tumour development¹⁵. To study *mdm2* function *in vivo*, we generated an *mdm2* null allele by homologous recombination. *Mdm2* null mice are not viable, and further analysis revealed embryonic lethality around implantation. To examine the importance of the interaction of MDM2 with *p53* *in vivo*, we crossed mice heterozygous for *mdm2* and *p53* and obtained progeny homozygous for both *p53* and *mdm2* null alleles. Rescue of the *mdm2*^{−/−} lethality in a *p53* null background suggests that a critical *in vivo* function of MDM2 is the negative regulation of *p53* activity.

The *mdm2* gene was cloned from a murine 129 library and characterized (Fig. 1). To mutate the *mdm2* gene in mouse embryonic stem (ES) cells, we generated a vector that deletes two-thirds of the MDM2 protein-coding sequences, including the acidic and zing-finger-like domains¹⁶ (amino acids 221–489) by replacing them with a neomycin-resistance expression cassette (Fig. 1a, b). Correctly targeted clones were detected by the presence of an additional 5.2-kb band using either 5' or 3' probes external to the region of vector homology (Fig. 1c). One correctly targeted ES clone successfully contributed to the germ line of chimaeric mice generated by blastocyst injection.

Mice heterozygous for the *mdm2* deletion allele appeared phenotypically normal and were fertile. However, no homozygous mutants were identified among more than 50 offspring born from matings between heterozygotes, indicating that *mdm2* mutant mice died during embryogenesis. Therefore, embryos from timed matings between heterozygotes were analysed at different stages of gestation (Table 1a). Empty deciduae were commonly

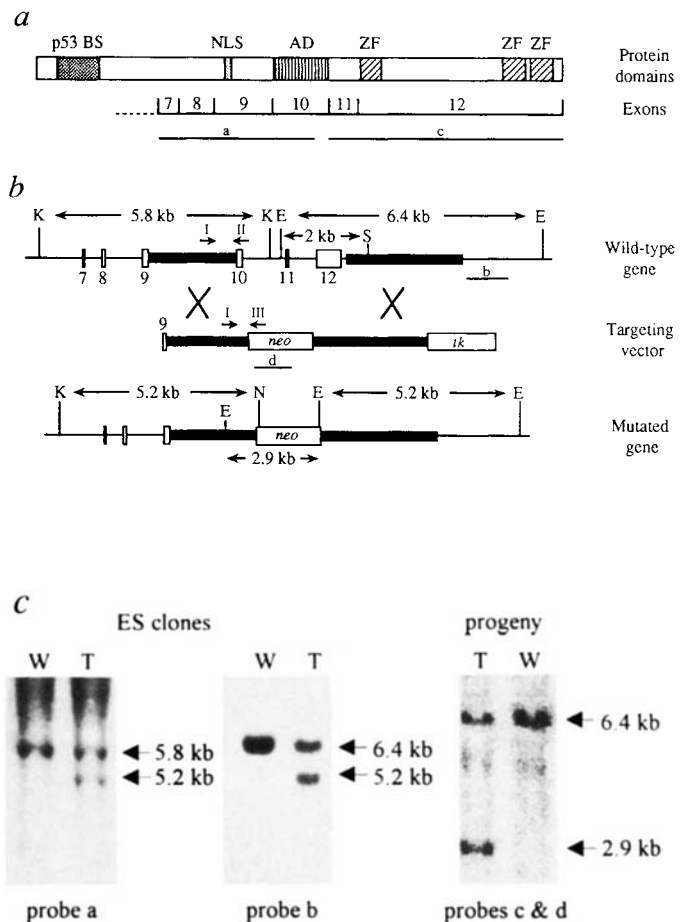


FIG. 1 Disruption of the *mdm2* gene. **a**, The functional domains of the MDM2 protein and exons 7–12 encoding those domains. **b**, Partial exon/intron structure of the *mdm2* gene, the targeting vector, and predicted homologous recombination events. **c**, Southern blot analysis of genomic DNA isolated from the ES cell clone that resulted in germline transmission, and offspring from heterozygous matings. DNA from ES cell clones was digested with *KpnI* and *NotI* and hybridized with probe a (left) or digested with *EcoRI* and hybridized with probe b (middle). DNA from progeny was digested with *EcoRI* and hybridized with probes c and d (right). Abbreviations: p53 BS, p53 binding site; NLS, nuclear localization signal; AD, acidic domain; ZF, zinc-finger; K, *KpnI*; N, *NotI*; E, *EcoRI*; S, *StuI*; kb, kilobases; W, wild type; T, targeted. Roman numerals denote primers used in PCR amplification. Lines identified by lower-case letters indicate probes used in Southern analysis.

METHODS. A 129/SvEv mouse genomic library was screened with a PCR-amplified *mdm2* cDNA probe. Three overlapping phage clones were obtained that spanned the entire *mdm2* gene consisting of 12 exons dispersed in approximately 20 kb of DNA as determined by Southern analysis, PCR amplification and sequencing (R.M.L., unpublished observations). A PGKneobpA resistance expression cassette was inserted in reverse orientation relative to the direction of *mdm2* transcription²⁵. A MC1tkpA herpes simplex virus thymidine kinase expression cassette was added to the long arm of homology to enrich for homologous recombinants using negative selection with 1-(2-deoxy-2-fluoro-b-D-arabinofuranosyl)-s-iodouracil (FIAU)²⁶. Linearized vector (25 µg) was electroporated into 10⁷ AB-1 ES cells that were subsequently cultured in the presence of G418 and FIAU^{25,27}. When the vector is recombined with the endogenous *mdm2* gene, new *NotI* and *EcoRI* sites are introduced. G418/FIAU-resistant ES clones (1000) were initially screened by Southern blot analysis using probe a. Correctly targeted clones were expanded for further Southern blot analysis using probe b. Six correctly targeted ES clones were identified. Three of the *mdm2* mutant ES clones were microinjected into C57BL/6J blastocysts and transferred to the uterine horn of day 2.5 pseudopregnant females. Resulting chimaeras were bred to C57BL/6J mice. One of three mutant ES cell lines injected contributed to the germ line of mice.

† To whom correspondence should be addressed.

TABLE 1 Genetic analysis of mutants

(a) <i>mdm2</i> mutant embryos							
Stage	Phenotypes				Genotypes		
	<i>mdm2</i> ^{+/+} × <i>mdm2</i> ^{+/+}		<i>mdm2</i> ^{+/+} × <i>mdm2</i> ^{+/-}		<i>mdm2</i> ^{+/+} × <i>mdm2</i> ^{+/+}		<i>mdm2</i> ^{-/-}
	Normal	Abnormal*	Normal	Abnormal	<i>mdm2</i> ^{+/+}	<i>mdm2</i> ^{+/-}	
E10.5	19	0	6	4	2	4	0
E 8.5	30	1	38	7	4	17	0
E 7.5	33	2	49	14	7	9	0
E 6.5	9	0	11	5	1	4	0
Total	91	3	104	30	14	34	0

(b) <i>mdm2/p53</i> mutant mice							
<i>p53</i> ^{+/+} <i>mdm2</i> ^{+/+} × <i>p53</i> ^{+/+} <i>mdm2</i> ^{+/+}				<i>p53</i> ^{+/+} <i>mdm2</i> ^{-/-} × <i>p53</i> ^{+/+} <i>mdm2</i> ^{+/+}			
Genotype		No. of mice		Genotype		No. of mice	
<i>p53</i> ^{+/+}	<i>mdm2</i> ^{+/+}	3		<i>p53</i> ^{+/+}	<i>mdm2</i> ^{+/+}	12	
<i>p53</i> ^{+/+}	<i>mdm2</i> ^{+/+}	5		<i>p53</i> ^{+/+}	<i>mdm2</i> ^{-/-}	0	
<i>p53</i> ^{+/+}	<i>mdm2</i> ^{-/-}	0		<i>p53</i> ^{+/+}	<i>mdm2</i> ^{+/-}	9	
<i>p53</i> ^{+/-}	<i>mdm2</i> ^{+/+}	7		<i>p53</i> ^{-/-}	<i>mdm2</i> ^{-/-}	9	
<i>p53</i> ^{+/-}	<i>mdm2</i> ^{+/-}	11					
<i>p53</i> ^{+/+}	<i>mdm2</i> ^{-/-}	0					
<i>p53</i> ^{-/-}	<i>mdm2</i> ^{+/+}	1					
<i>p53</i> ^{-/-}	<i>mdm2</i> ^{+/-}	7					
<i>p53</i> ^{-/-}	<i>mdm2</i> ^{-/-}	2					

a, Genotypic analysis of *mdm2* in embryos generated from heterozygous crosses. E10.5 embryos were analysed by Southern blots using probes c and d (Fig. 1). The remaining embryos were genotyped by polymerase chain reaction (PCR) using a three-primer combination: I, 5'-TGTGGCTGGAGCATGGGTATTG-3'; II, 5'-ATCTGAGAGCTCGTCCCTTCG-3'; III, 5'-GGCGGAAAGAACAGCTGGGGC-3'. I and II amplify the wild-type *mdm2* allele (415 bp), and I and III amplify the mutant allele (284 bp), as is shown in Fig. 3a, b. Analysis of *mdm2* and *p53* genotypes in offspring generated from *mdm2/p53* crosses as indicated. The genotypes were determined as shown in Fig. 3.

* The abnormal phenotype is defined as an empty decidua.

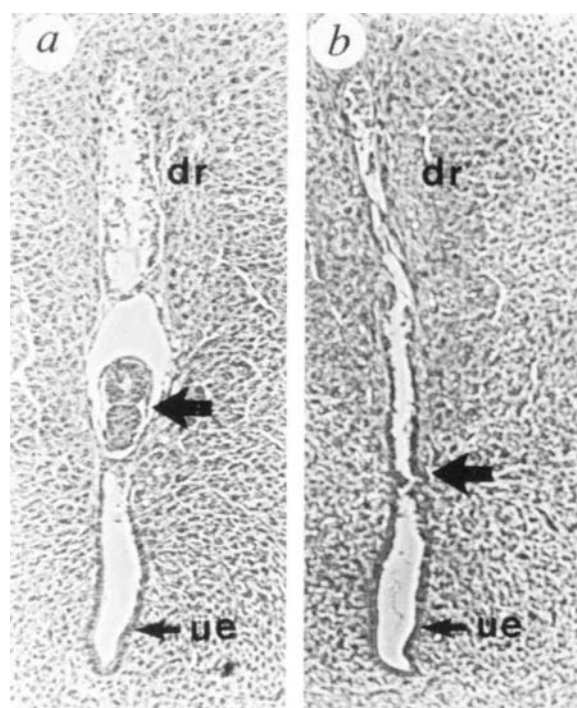


FIG. 2 Histological analysis of E5.5 embryos from a cross between two *mdm2* heterozygous mutant mice. a, Transverse section through the decidua of a normal embryo. b, Transverse section through a decidua of an abnormal E5.5 embryo. Large arrows mark the location of the embryo. Abbreviations: ue, uterine epithelium; dr, decidua reaction.

METHODS. Embryos were processed for histological analysis by fixation in Bouin's fixative, dehydrated and embedded in paraffin. Sections 6 μ m thick were cut and stained with haematoxylin and eosin.

seen in these crosses: of a total of 134 deciduae analysed, 30 (22%) were empty. In contrast, in crosses between *mdm2* heterozygotes and normal mice, this abnormality was seen in only 3% of the deciduae analysed. We did not find any *mdm2* homozygous mutant embryos from days E6.5–E10.5 (Table 1a). Blastocysts were then isolated from a heterozygous *mdm2* mutant female that had been mated with a heterozygous *mdm2* mutant male. One of the eight blastocysts examined contained no wild-type *mdm2*. Our identification of a viable *mdm2* null blastocyst and the lack of *mdm2* null embryos indicates that the time of death must be between implantation and E6.5.

To examine the phenotype in more detail, we isolated, fixed and paraffin embedded the deciduae of an *mdm2*^{+/+} female crossed with an *mdm2*^{+/+} male 5.5 days post-coitum. The embryos were sectioned and, although they could not be genetically typed, the two phenotypes were obvious, one normal and the other abnormal (Fig. 2). The abnormal embryo had few, if any, cells (Fig. 2b, large arrow) compared with a normal embryo (Fig. 2a, large arrow), but had formed an active site of implantation, visible by the decidual reaction, thus explaining the presence of empty deciduae at later stages of development.

One of the functions of MDM2 identified in tissue-culture experiments is the negative regulation of *p53* growth suppression¹⁷ and transcriptional activation^{12–14}. Indeed, *p53*, *p21* and *mdm2* are highly expressed in ES cells as compared with normal tissues by northern analysis, which indicates expression early in development (data not shown). We therefore hypothesized that the embryonic lethality seen in *mdm2* homozygous mutants was due to an inability to downregulate *p53* function, which could lead to growth arrest or apoptosis. Because a *p53* null mouse is viable^{10,11}, we tested our hypothesis by interbreeding mice heterozygous for both *mdm2* and *p53* genes. Strikingly, genotyping of offspring revealed the presence of *mdm2* homozygous mutants in a *p53* null background (Fig. 3a, b). From the mendelian ratio, one of 16 mice from this cross could be expected to be a double homozygote. Of 36 progeny born from matings of double heterozygote crosses, two were homozygous for both *mdm2* and *p53* mutant genes (Table 1b). In addition, no homozygous mutant *mdm2* mice were identified in either a wild-type

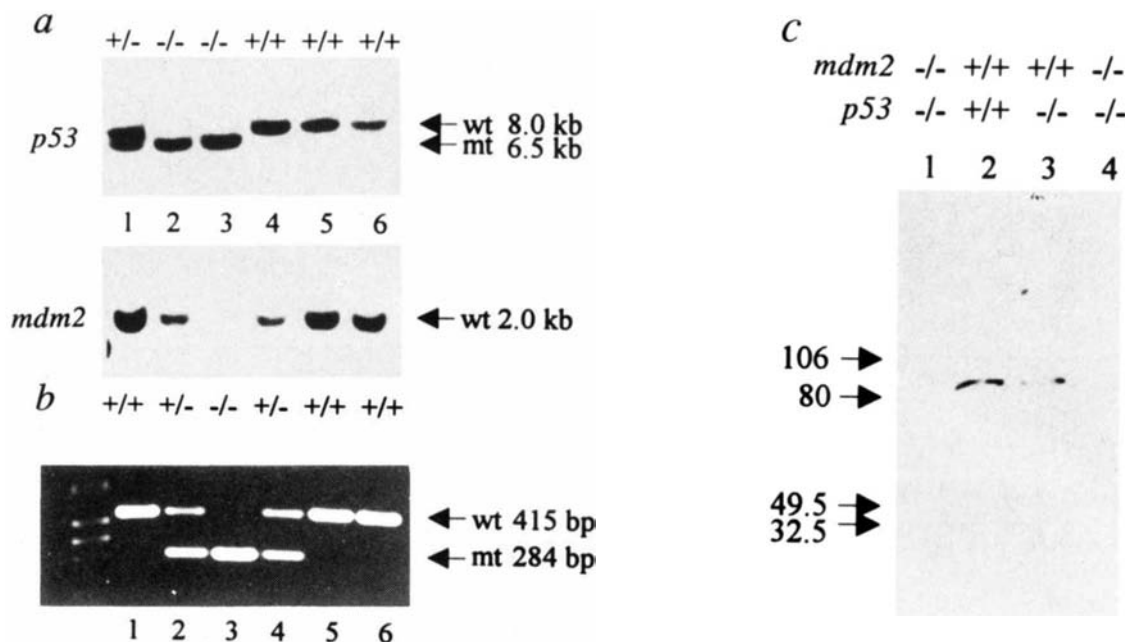


FIG. 3 *a* and *b*, Southern blot and PCR analysis of progeny from matings between mice heterozygous at both *mdm2* and *p53* alleles. The mice heterozygous for the *mdm2* mutation created in this study were mated with *p53* null mice (Jackson laboratory)¹¹. *a*, The progeny heterozygous at both genes were mated, and their progeny analysed for the homozygous deletion of *mdm2* by Southern blot using probe *c* (Fig. 1). The same blots were analysed for the *p53* null allele using a probe as described¹¹. *b*, The *mdm2* genotypes of the mice were also checked by PCR amplification using the primers described in Table 1a. *c*, Western blot analysis of *mdm2* mutants. Total protein was extracted from the uteri of *p53*^{-/-} *mdm2*^{-/-} (lanes 1 and 4), *p53*^{+/+} *mdm2*^{+/+} (lane 2), and *p53*^{-/-} *mdm2*^{+/+} mice (lane 3). We chose the uterus because it contains high levels of MDM2 (S. de

Rozieres and G.L., unpublished observations). The position of *M_r* markers is shown on the left.

METHODS. For the western blot, total protein extracted (50 µg) from the uteri of mice was resolved by electrophoresis through an SDS-7.5% polyacrylamide gel. Low-range prestained *M_r* standards were also used (Bio-Rad). Blots were performed using an MDM2 antibody prepared as follows: the *mdm2* cDNA was cloned into pRSETB (Invitrogen), which added a His tag hexamer. MDM2 was purified by immobilized metal affinity chromatography and used as an antigen in rabbits. The blots were developed using horseradish peroxidase as the secondary antibody and the ECL method (Amersham). Fast green staining of the blots confirmed that equivalent amounts of protein were present in each lane.

or heterozygous *p53* background, indicating that heterozygosity of *p53* is insufficient to rescue the embryonic lethality of *mdm2* nulls. To further substantiate this finding, males that were homozygous mutant at both loci were mated with females that were heterozygous at both loci. Of viable mice born, 30% had homozygous deletions in both genes (Table 1b). The ratio is slightly skewed because one-quarter of the animals are expected to be homozygous for *mdm2* and heterozygous for *p53* and should therefore be expected to die *in utero*.

To show that an abnormal MDM2 polypeptide was not generated, we performed western blot analysis of tissues from *mdm2*/*p53* mutant homozygous mice. Although wild-type *mdm2* mice, regardless of their *p53* status, contained a protein of the expected relative molecular mass (*M_r*, 90K), the *mdm2*/*p53* null mutant mice did not make the p90 MDM2 polypeptide (Fig. 3c). Exposure of several western blots for longer times did not reveal the presence of a small peptide, although its existence cannot be completely ruled out.

Our *in vivo* analyses demonstrated a tight functional relationship between *p53* and MDM2 in early development. Our first important observation was that the loss of *mdm2* resulted in early embryonic lethality. The embryos died between implantation and 5.5 days of development. Within eight cell divisions, the approximate number of divisions achieved by 5.5 days of development, the null embryo was no longer recognizable as such. This lethal event is one of the earliest seen by the deletion of a gene affecting proliferation. Because MDM2 is a negative regulator of *p53* function¹²⁻¹⁴, we suspect that *p53* function was not inactivated in our embryos. Thus, although loss of *p53* caused no problems for the embryo, the absence of its negative

regulator was lethal. Because stabilization of *p53* leads to growth arrest or apoptosis after DNA damage^{5,7,18-22}, it is possible that either of these mechanisms might cause embryonic death in the *mdm2* null mice. The early lethality of the *mdm2*^{-/-} mice precludes the observation of any later effects. Further, our results emphasize the importance of assaying *p53* functionally, as the physiological ratio of *p53* to MDM2 will influence *p53* activity.

The second major observation is that the *mdm2* null phenotype was rescued by deletion of *p53*: double-homozygous-mutant mice were viable. Subtle abnormalities may exist, however, as the *mdm2*^{-/-} *p53*^{-/-} and *mdm2*^{+/+} *p53*^{-/-} females had few litters and very few pups in their litters. These data imply a function for MDM2 in addition to inactivation of *p53*. Indeed, interactions between MDM2 and the retinoblastoma protein have recently been identified²³. Furthermore, interaction of MDM2 with E2F1 and DPl augments their transcriptional activation of genes involved in S-phase progression²⁴.

Our results provide genetic evidence for the interaction of MDM2 and *p53* *in vivo*, and also indicate that the *mdm2* null embryos die because *p53* function cannot be downregulated. Investigation of viable double-null homozygotes might allow us to determine whether the loss of *mdm2* in mice affects the timing and range of tumour development in the *p53* null background. □

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Rescue of embryonic lethality in Mdm2-deficient mice by absence of p53

Stephen N. Jones^{*†}, Amy E. Roe^{*†},
Lawrence A. Donehower[†] & Allan Bradley^{*†}

^{*} Department of Molecular and Human Genetics, [†] Howard Hughes Medical Institute, and [‡] Department of Molecular Virology, Baylor College of Medicine, One Baylor Plaza, Houston, Texas 77030, USA

THE *Mdm2* proto-oncogene was originally identified as one of several genes contained on a mouse double minute chromosome present in a transformed derivative of 3T3 cells¹. Overexpression of *Mdm2* can immortalize primary cultures of rodent fibroblasts². Human *MDM2* is amplified in 30–40% of sarcomas, and is overexpressed in leukaemic cells^{3,4}. The *Mdm2* oncoprotein forms a complex with the p53 tumour-suppressor protein and inhibits p53-mediated transregulation of gene expression^{5,6}. Because *Mdm2*

expression increases in response to p53, *Mdm2*–p53 binding may autoregulate *Mdm2* expression and modulate the activity of p53 in the cell^{7,8}. We have created *Mdm2*-null and *Mdm2*/p53-null mice to determine whether *Mdm2* possesses developmental functions in addition to the ability to complex with p53, and to investigate the biological role of *Mdm2*–p53 complex formation in development. Mice deficient for *Mdm2* die early in development. In contrast, mice deficient for both *Mdm2* and p53 develop normally and are viable. These results suggest that a critical role of *Mdm2* in development is the regulation of p53 function.

Mdm2 was cloned from a 129-strain genomic library and the gene structure was characterized to allow the creation of a replacement vector for gene targeting in embryonic stem (ES) cells (Fig. 1a). Positive and negative selection of transfected AB2.1 ES cells led to the isolation of one clone which had replaced the 7.1-kilobase (kb) region of *Mdm2* which encodes all putative transcription function motifs of *Mdm2* with an *hprt* reporter gene. Injection of the targeted ES cells into host blastocysts gave rise to chimaeric mice which transmitted the mutated *Mdm2* allele (*mdm2*^{ml}) to F₁ progeny (Fig. 1b). Intercrosses between mice heterozygous for the targeted allele (*mdm2*^{ml}/+) were performed and the offspring genotyped by Southern ana-

FIG. 1 Gene targeting in *Mdm2*.

a, Structure of *Mdm2* gene and targeting vector. A replacement targeting vector containing 1.6 kb of 5' homology and 2.2 kb of 3' homology was constructed with markers for positive selection (*hprt* mini-gene)²⁰ and negative selection (thymidine kinase)²¹. b, Southern analysis of targeted ES cell DNA (lane 1), and genomic DNA from a wild-type F₁ progeny mouse (lane 2) and mutant F₁ progeny mouse (lane 3). The presence of a 5.7 kb *Bam*HI band following hybridization with a 5' probe, and the presence of a 4.7 kb *Eco*RI band following hybridization with a 3' probe, indicates proper targeting of the *Mdm2* gene in the ES clone by pMdmKO1.

METHODS. The entire *Mdm2* gene was cloned from a 129-strain mouse genomic phage library and characterized by digestion with endonuclease restriction enzymes, including *Bam*HI (HI) and *Eco*RI (RI). A 1.6 kb *Kpn*I–*Eco*RI fragment and a 2.2 kb *Pst*I fragment which flank a 7.1 kb region of *Mdm2* encoding all of the putative transcription factor motifs were used to create the gene replacement vector pMdmKO1. AB2.1 ES cells²⁰ were electroporated with pMdmKO1 and placed under selection. Mini-Southern analysis²² of the resulting 500 colonies identified one clone which exhibited proper targeting at the 3' end. This clone was expanded and analysed by Southern blot hybridizations using a 1.3 kb, *Bam*HI–*Kpn*I 5' probe and a 350 bp, *Sac*I–*Sal*I 3' probe. Injection of these ES cells into host blastocysts yielded chimaeric mice that were capable of transmitting the mutated allele to F₁ progeny.

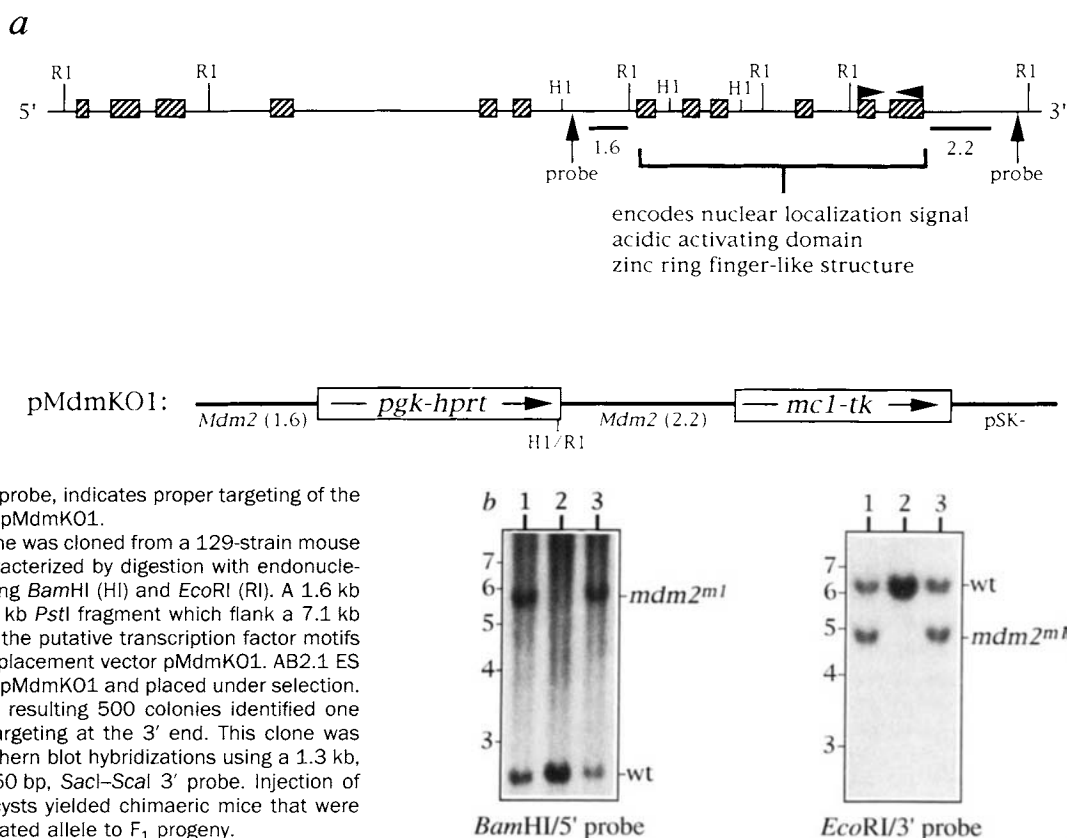


TABLE 1 Analysis of embryos from *mdm2^{ml}/+* intercrosses

Gestational age	Total number	Reabsorptions	Genotyped	<i>mdm2^{ml}/mdm2^{ml}</i>
day 9.5	20	6	14	0
day 8.5	25	6	19	0
day 7.5	29	8	21	0

Embryos were collected from female partners of *mdm2^{ml}/+* intercrosses on day 9.5, 8.5 or 7.5 of gestation. The total number of embryos and the number of embryos undergoing maternal reabsorption are given. Those not undergoing reabsorption were genotyped by PCR using an *Mdm2* primer pair which amplifies a region of genomic DNA between exons 11 and 12 (see horizontal arrows in Fig. 1a). This region of the *Mdm2* gene is deleted following homologous recombination of the gene with the targeting vector. A pair of primers to exon 2 of the *Wnt-5a* gene were present in each reaction to act as a control for the thermocycling reactions and for integrity of the starting template DNA. All of the embryos tested contained DNA that could be amplified using the *Mdm2* primers, indicating that none of the embryos were null for *Mdm2* (*mdm2^{ml}/mdm2^{ml}*).

lysis of genomic DNA isolated from tail biopsy. Of the 109 progeny obtained from intercrosses of *mdm2^{ml}/+* mice, 33% were wild-type for *Mdm2* (+/+) and 67% were heterozygous for the targeted *Mdm2* allele (*mdm2^{ml}/+*). No viable *mdm2^{ml}/mdm2^{ml}* mice were detected in these crosses, suggesting that absence of functional Mdm2 leads to embryonic lethality. A strategy based on polymerase chain reaction (PCR) was used to determine the gestational time of embryonic lethality of *mdm2^{ml}/mdm2^{ml}* mice. Genomic DNA was isolated from the yolk sacs of embryos or from whole embryos collected at various times during development from female partners of *mdm2^{ml}/+* intercrosses. Coamplification of a region deleted in *mdm2^{ml}* and of a control region in *Wnt-5a* was performed to detect the presence of *mdm2^{ml}/mdm2^{ml}* embryos. The results indicate that *mdm2^{ml}/mdm2^{ml}* embryos die before day 7.5 of gestation (Table 1). The large percentage (28%) of embryos found to be in the latter stages of reabsorption on day 7.5 suggests that embryonic demise occurs after implantation of the embryo in the wall of the uterus at day 4.5 but before day 7.5 of gestation.

To examine the developmental defect in Mdm2-deficient embryos, histological analysis of embryos collected from female partners of *mdm2^{ml}/+* intercrosses was performed. Approximately one-quarter (5 of 19) of the embryos examined at day 6.5 of gestation exhibited an abnormal embryonic architecture (Fig. 2). Very little embryonic material was present in the maternal decidua, and the most developed embryo had not progressed significantly beyond day 5.5 of gestation. The time of the developmental block in these presumptive mutants coincides with the sudden increase in cell cycle rate that occurs at day 5.5–6.0 of mouse development⁹.

In situ hybridization of wild-type day-7.5 embryo sections indicates that *Mdm2* is ubiquitously expressed in the developing embryo (data not shown). On day 8.5 of development, *p53* is also expressed ubiquitously¹⁰. To confirm that both *Mdm2* and *p53* are expressed on day 6.0–6.5 of gestation, RNA was isolated from wild-type egg cylinder stage embryos and reverse transcribed into complementary DNA. PCR amplification using oligonucleotide primers for *Mdm2* and *p53* indicates that both transcripts are present when the presumptive mutants die (Fig. 3a). These findings are consistent with the proposal that Mdm2 functions to regulate *p53* activity in the cell. To examine whether the developmental defect in Mdm2-null mice results from alterations in Mdm2-mediated gene expression, or is due to a loss in the ability of Mdm2 to regulate *p53* activity, *mdm2^{ml}/+* and *p53 -/-* mice¹¹ were intercrossed to produce mice which were *mdm2^{ml}/+*, *p53 +/-*. These compound heterozygous mice were mated and the resulting progeny genotyped for *Mdm2* and *p53* status by Southern analysis (Fig. 3b). Nine viable *mdm2^{ml}/mdm2^{ml}* mice were obtained from this cross, all of which were

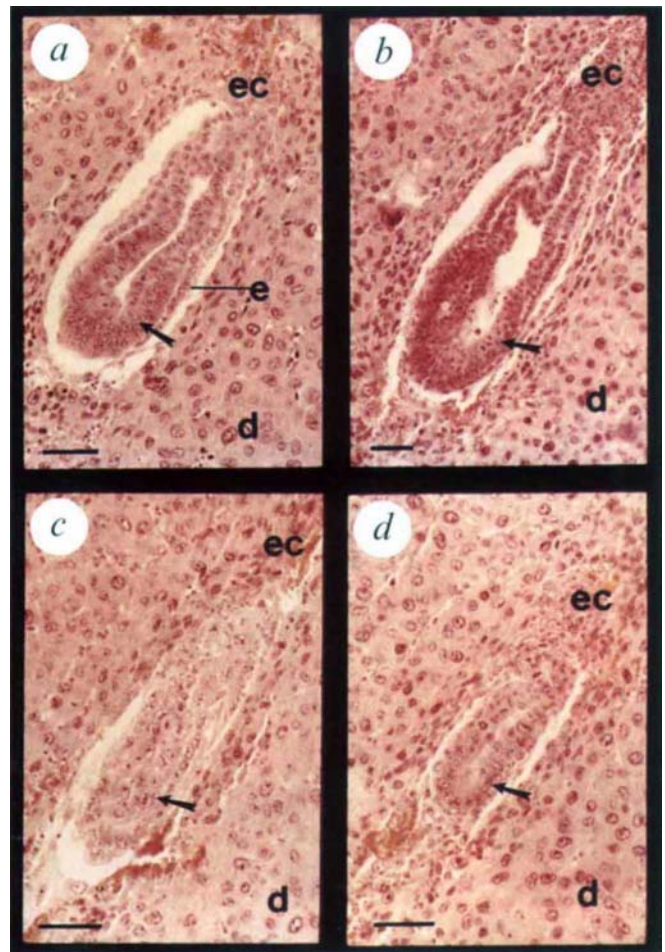


FIG. 2 Histology of embryos from *mdm2^{ml}/+* intercrosses on day 6.5 of gestation. a, b, Sagittal sections of phenotypic wild-type embryos exhibiting well-organized embryonic ectoderm (arrows). c, d, Sagittal sections of mutant embryos. The embryos contain fewer embryonic ectoderm cells, lack a normal wild-type architecture, and are smaller in size than the wild-type embryos. Abbreviations: d, maternal decidua; ec, ectoplacental cone; e, endoderm. Scale bar, 50 μ m.

METHODS. Embryos were collected from female partners of *mdm2^{ml}/+* intercrosses on day 6.5 post-coitum and fixed in Bouin's solution. Embedding, sectioning and staining of the embryo sections with haematoxylin and eosin were performed as described previously²³.

p53 -/-. In addition, matings between *mdm2^{ml}/+*, *p53 +/-* and *mdm2^{ml}/+*, *p53 -/-* mice have yielded 7 *mdm2^{ml}/mdm2^{ml}* mice, all of which were *p53 -/-*. The *mdm2^{ml}/mdm2^{ml}*, *p53 -/-* mice were recovered at the expected ratios in the two crosses, accounting for the non-viability of the *mdm2^{ml}/mdm2^{ml}* embryos on the *p53 +/-* and *p53 +/-* background. Furthermore, 17 double-null mice have been recovered from *mdm2^{ml}/mdm2^{ml}*, *p53 -/-* intercrosses. Thus absence of *p53* rescues the embryonic lethality seen in Mdm2-deficient mice. These results indicate that Mdm2 functions in development primarily as a regulator of *p53* activity. Although these results do not exclude the possibility that Mdm2 possesses other functions, these functions cannot be critical in development because Mdm2/*p53* double-null mice are developmentally normal, fertile and appear phenotypically indistinguishable from wild-type mice.

The mutation created in *Mdm2* does not alter the 5' portion of the gene which encodes the Mdm2 domain that binds with *p53* (ref. 12). It is conceivable that a truncated Mdm2 protein might be produced from the targeted allele that could bind with *p53* and induce lethality in mice. This lethality would then be rescued in mice nullizygous for *p53*. To test this possibility, RNA

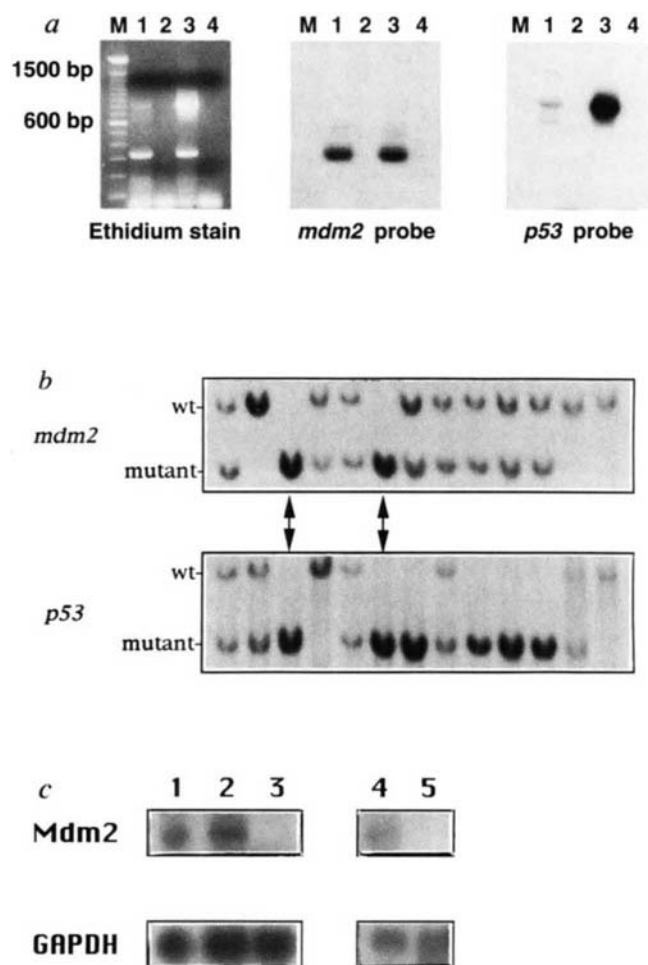


FIG. 3 a, Reverse transcription–PCR amplification (RT–PCR) of RNA from day 6.0–6.5 wild-type embryos. Oligonucleotide primers for both *Mdm2* and *p53* cDNA were present in the PCR reaction. The larger (840 bp) and smaller (344 bp) fragments observed after staining of the gel with ethidium bromide (left) are amplification products of *p53* and *Mdm2* cDNA, respectively. Lane 1, RT–PCR products of day 6.0–6.5 embryo RNA. Lane 2, as lane 1 except no reverse transcriptase was present in the RT–PCR reaction. Lane 3, RT–PCR products of wild-type primary embryo fibroblast RNA. Lane 4, PCR reaction performed in the absence of template. Lane M is a 100-bp DNA size standard. Southern analysis of the PCR products using either an *Mdm2*-specific oligonucleotide probe (middle) or a *p53*-specific oligonucleotide probe (right) was performed to confirm the identity of the PCR fragments. b, Southern analysis of progeny of *mdm2*^{ml}/+, *p53* +/- intercrosses. Compound heterozygous mice were mated and the offspring genotyped for *Mdm2* (top) and *p53* (bottom) by Southern analysis. Two of the mice were determined to be *mdm2*^{ml}/*mdm2*^{ml}. These two mice were also *p53* -/- (double-headed arrows). c, Northern analysis of total RNA isolated from the kidney and/or spleen of mice which were either wild-type (lane 1), *p53* -/- (lanes 2 and 4), or *mdm2*^{ml}/*mdm2*^{ml}, *p53* -/- (lanes 3 and 5). Top, *Mdm2* cDNA probe; bottom, *GAPDH* probe, used as a control for load and integrity of RNA. No *Mdm2* transcript is detected in the *Mdm2*, *p53*-double-null tissue.

METHODS. Total RNA was isolated from primary embryo fibroblasts for RT–PCR and from whole tissue for either RT–PCR or northern analysis using RNAzol B as described by the manufacturer (Tel-Test, Friendswood, TX). RT–PCR was performed using the Superscript Pre-amplification System (Gibco-BRL, Bethesda, MD) followed by a 40-cycle PCR using *Mdm2* primers (5'-ATGTGCAATACCAACATCTCTGTGTC-3' and 5'-GCTGACTTACAGCCACTAAATTTC-3') and *p53* primers (5'-CTGCAGTCTGGGACAGCCAAGTC-3' and 5'-GTCAGTCAGACTCAGTCCGGGGT-3'). Of each sample, 40% was visualized on a 2% agarose-ethidium bromide gel. Southern analysis was performed on the PCR products using an oligonucleotide probe which hybridizes to either *Mdm2* or *p53* coding sequences which are internal to the primers used in the PCR reaction. Southern analysis was performed on genomic DNA isolated from tail biopsies of four-week-old mice. The DNA was digested with *EcoRI*, and the Southern blot probed with the *Mdm2* *SacI*–*Scal* 3' probe described in Fig. 1. After autoradiography, the blot was stripped and re-probed with a *p53* exon 11 probe that recognizes a 16-kb *EcoRI* fragment in wild-type DNA, and an 8-kb *EcoRI* fragment in the mutated *p53* gene¹². The double-headed arrows indicate genomic DNA of mice which are both *Mdm2*- and *p53*-null.

was isolated from the kidney and spleen of mice which were either wild type, *p53*-deficient, or *p53*- and *Mdm2*-deficient. Northern analysis failed to detect any *Mdm2* transcripts in *mdm2*^{ml}/*mdm2*^{ml} tissue (Fig. 3c). Furthermore, *mdm2*^{ml}/+ mice that are wildtype for *p53* are viable. Thus it is unlikely that a hypothetical truncated *Mdm2*–*p53* complex causes the embryonic lethality seen in *mdm2*^{ml}/*mdm2*^{ml} mice.

The *p53* protein has been shown to regulate cell growth negatively by arresting cells late in the G1 phase of the cell cycle^{13,17}. In resting cells stimulated with serum, the levels of *Mdm2* and *Mdm2*–*p53* complex rise late in G1, suggesting that *Mdm2* can downregulate the ability of *p53* to block progression of the cell cycle¹⁸. More recently, overexpression of exogenous *Mdm2* in

transfected cells has been found to inhibit the ability of these cells to undergo *p53*-mediated growth arrest following treatment with ionizing radiation, providing direct evidence that *Mdm2* is involved in inhibiting *p53* function in a known pathway¹⁹. Our data indicate that *Mdm2* plays an important role in development by inhibiting *p53* function during a period in gestation when the mean cell cycling time of primitive ectoderm decreases dramatically in normal embryos⁵. Thus the developmental defect in *Mdm2*-null mice might arise as a result of failure to inhibit *p53*-mediated suppression of cell cycling during this time of rapid cell division. This finding might also explain the difficulty experienced by researchers attempting to construct transgenic mice which overexpress wild-type *p53* (A. Bernstein and J. Butel, personal communication). □

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Movement and force produced by a single myosin head

J. E. Molloy, J. E. Burns, J. Kendrick-Jones*,
R. T. Tregear* & D. C. S. White

Department of Biology, University of York, York YO1 5DW, UK

*Laboratory of Molecular Biology, Hills Road,
Cambridge CB2 2QH, UK

MUSCLE contraction is driven by the cyclical interaction of myosin with actin, coupled to the breakdown of ATP. Studies of the interaction of filamentous myosin¹ and of a double-headed proteolytic fragment, heavy meromyosin (HMM)^{2,3}, with actin have demonstrated discrete mechanical events, arising from stochastic interaction of single myosin molecules with actin. Here we show, using an optical-tweezers transducer^{2,4}, that a single myosin subfragment-1 (S1), which is a single myosin head, can act as an independent generator of force and movement. Our analysis accounts for the broad distribution of displacement amplitudes observed, and indicates that the underlying movement (working stroke) produced by a single acto-S1 interaction is ~4 nm, considerably shorter than previous estimates^{1-3,5} but consistent with structural data⁶. We measure the average force generated by S1 or HMM to be at least 1.7 pN under isometric conditions.

Previous studies⁷⁻⁹, under conditions in which several molecules are interacting with the actin filament, indicated that both S1 and single-headed myosin are capable of generating movement and force. To demonstrate that such events occur between a single myosin head and an actin monomer, we have measured interactions between S1 and actin using an optical-tweezers apparatus⁵; its means of operation is similar to that used previously², except that it synthesizes a double trap by rapid scanning of a single laser beam, and has a position detector with a frequency response high enough (2.2 kHz) to allow observation of the full amplitude of brownian motion.

An actin filament was suspended between two polystyrene beads held in optical traps, and its intermittent interaction with S1 or HMM bound to a stationary glass sphere at low surface density was monitored by measuring the motion of one of the beads (see Fig. 1 legend). The surface concentration of S1 or HMM and the bulk concentration of ATP were chosen so that mechanical interactions between a single S1 head and the actin filament could be readily observed.

When low concentrations of either S1 or HMM were used, fast-rising displacements could be clearly distinguished from thermal vibration of the actin filament (Fig. 1a-c). Most of the displacements were in one direction, but negative displacements were also observed (down arrows). By rotating the two trap positions through 180° about the myosin contact, so that the polarity of the actin filament was reversed, we found that the preferred direction of the displacements was also reversed and that ~80% of displacements occurred in the preferred direction. In 10 µM ATP, the average duration of positive and negative displacements was 90 ms and was independent of the amplitude of the observed attachment (Fig. 1g), suggesting that the presence of a preferred direction for attachment is not a kinetic effect arising from differences in probability of detachment, but is probably due to a myosin work stroke with its polarity determined by that of the actin filament. Lowering the ATP concentration from 10 µM (Fig. 1b) to 2 µM (Fig. 1c) increased the mean duration. Those amplitudes of positive and negative displacements which could be distinguished from thermal noise were measured from the mean resting position. Histograms of the distribution of measured events are shown for both HMM and S1 in Fig. 2a, b. The striking feature of our data is the significant number of very large displacements (up to 30 nm) and negative displacements (down to -25 nm). Large-amplitude

positive displacements have previously been observed^{2,3}, especially at low optical trap stiffness, but negative displacements had not.

Noise of the displacement records is due to the thermal motion of the bead. During both positive and negative displacement events the amplitude of brownian noise was reduced, indicating that system stiffness had increased (Fig. 1a-c). Particularly in traces obtained at 5 and 2 µM ATP, periods of low noise were also observed without significant changes in mean displacement (Fig. 1c). S1 stiffness, κ_m , predicted from noise analysis during these periods, was similar irrespective of displacement ($\kappa_m - \kappa_s = 0.48$ pN nm⁻¹).

Using low variance of noise (Fig. 1d) as the criterion for attachment, we again measured the size of displacements produced by S1 and HMM relative to the mean rest position. In contrast to the method above, this technique enabled us to identify work strokes that drove the bead to near its mean position. The resulting histograms are shown in Fig. 2c, d.

In feedback (force) mode (Fig. 1 legend) the bead was held in a fixed position, the forces exerted by the molecular interactions being compensated by movement of the laser trap, allowing estimates of the isometric forces produced by single acto-myosin interactions (Fig. 3a). The mean force of the events was 1.7 pN and their duration was similar to that of the displacements. We found that HMM produced approximately the same amount of force as S1 (Fig. 2e, f).

Controlled, large-amplitude length changes were applied by imposing movement to the laser beam holding the bead imaged on the photodetector. The forces were measured from the movement of the laser beam required to produce the correct movement of the bead (Fig. 3b). The minimum stiffness of a single acto-myosin interaction was determined from the change in force required to produce a given displacement. Mean stiffness, measured from the gradient of length-tension diagrams, was 0.17 pN nm⁻¹ (Fig. 3c), much less than the above estimates from noise analysis, but stiffer than the trap stiffness used for our estimates of the working stroke, and similar to estimates of crossbridge stiffness in muscle fibres.

The similarity between the displacements and forces produced by S1 and HMM suggests that the same molecular process is generating the mechanical events found in both preparations. The possibility that the S1 measurements might arise from contaminant HMM is excluded by the absence of HMM in gels of the S1 preparation (Fig. 1 legend) and the fact that only three times more S1 was needed than HMM to produce these mechanical results. We conclude that a single myosin head can generate force and movement.

To extract the size of the working stroke from our data, the randomizing effect of thermal filament displacement has to be considered. We believe that crossbridge attachment will occur with equal intrinsic probability over the full range of the brownian motion of the actin filament. The start position of the myosin molecule relative to the bead rest position is arbitrary, certainly between different bead/actin filament preparations, and probably to some extent between records using any particular actin filament. The actin filament is free to rotate in an unconstrained fashion, so on average every actin monomer must be equivalent. The beads and actin filament would be expected to rotate over several seconds¹⁰ (that is, the period of a single recording). Thus dependence of attachment upon azimuthal orientation⁵ will be detectable only in recordings made with sufficient speed. Different recordings will have different azimuthal start points. Although summation of mechanical events will be very rare at the dilutions of protein used, there is likely to be more than one S1 head able to interact with the actin filament over the time scale of these experiments.

Our interpretation of the distribution of displacements in Fig. 2a, b is that crossbridge attachments occur at any axial position of the actin filament, and that the working stroke produces a displacement from that arbitrary starting point. The distribution

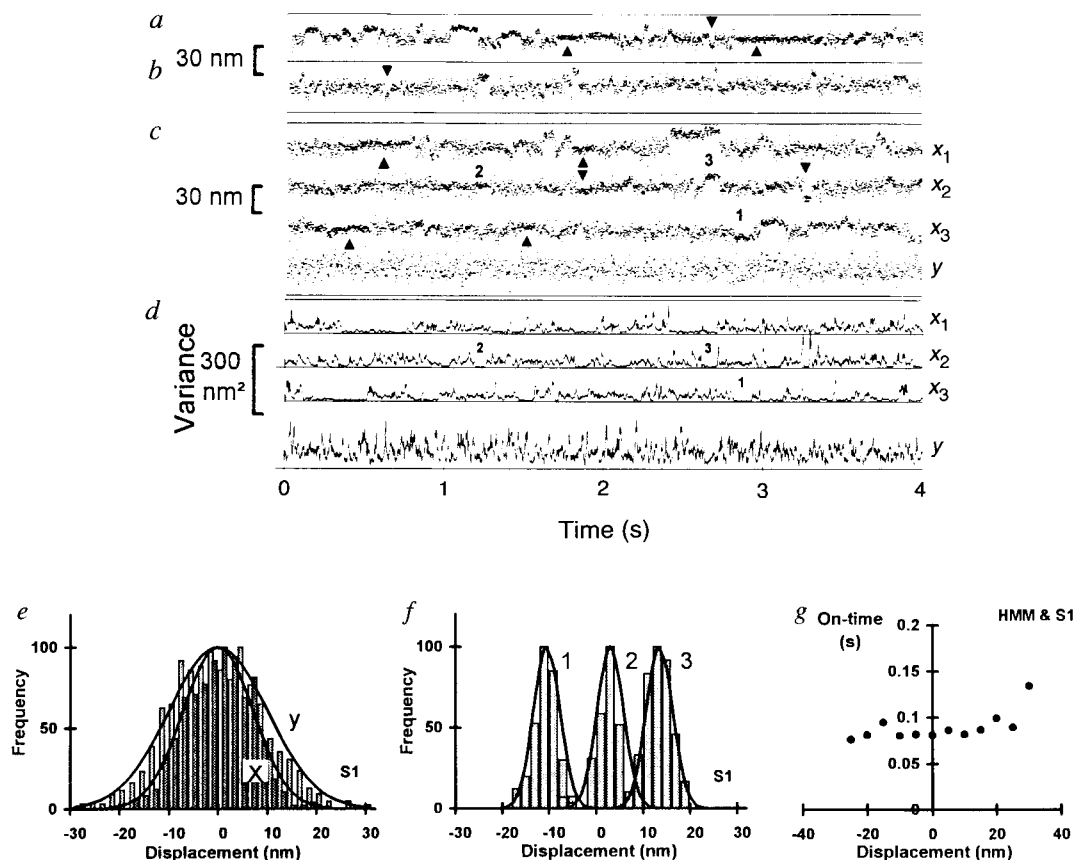


FIG. 1 Displacement of actin filament under near-zero load conditions resulting from interactions with low densities of *a*, HMM and *b–f*, S1. *a*, Record obtained on contact of HMM (bound to surface at $2 \mu\text{g ml}^{-1}$) with an actin filament in $10 \mu\text{M}$ ATP; κ_{trap} , 0.022 pN nm^{-1} . *b*, Similar record obtained with S1, at density of $5 \mu\text{g ml}^{-1}$, in $10 \mu\text{M}$ ATP; κ_{trap} , 0.021 pN nm^{-1} . *c*, Three sequential records (x_1 – x_3) obtained on contact of S1 heads, at $6 \mu\text{g ml}^{-1}$, with an actin filament in $2 \mu\text{M}$ ATP; κ_{trap} , 0.042 pN nm^{-1} . The 'noise' content of these records is due to residual brownian motion of the trapped bead. Detector noise was small by comparison (0.8 nm r.m.s.). Note the reduction in thermal vibration during the displacements. Negative displacements marked with down-pointing arrows. Periods showing long low thermal noise and little change of mean position are marked with up-pointing arrows. The fourth trace (*y*) is the record obtained in the *y*-axis during the sampling of x_1 . Sampling frequency 10 kHz , 5-point average. *d*, Running 13-point variance of the traces in *c*. The gridlines are the zero-axis for the trace immediately above that line. *e, f*, Histograms of the distribution of displacements during the records shown in *c*. *e*, The distribution in the *y*-axis and in the *x*-axis during a 'high-noise' section of the trace. The lines are Boltzmann distributions with zero mean and stiffness $\kappa_y = 0.04 \text{ pN nm}^{-1}$ and $\kappa_x = 0.08 \text{ pN nm}^{-1}$. The data indicate that the compliance in the *x*-direction is half that in the *y*-direction ($\kappa_x = 2 \kappa_y$ (see Methods)). This *y*-axis stiffness is very similar to that determined by other means for a single bead. *f*, The distribution during the 'low-noise' sections marked 1, 2 and 3 in *c*. The fitted curves assume a stiffness κ_{xm} of 0.56 pN nm^{-1} . To estimate the amplitude of brownian motion the detector bandwidth must be greater than the brownian noise bandwidth ($\sim 600 \text{ Hz}$; controlled by viscous drag on the bead and trap stiffness¹²). Our detector bandwidth (2.2 kHz) was sufficient to estimate the amplitude of brownian noise but gave poor phase information. We were therefore unable to determine accurately the start position of a bead immediately before a crossbridge interaction. *g*, Plot of attached lifetime (on-time) versus displacement during attachment. On-time is nearly independent of displacement polarity and magnitude.

METHODS. S1 was prepared by digestion of rabbit psoas muscle myosin with papain under conditions of low salt and high magnesium; the reaction was stopped with E64 and the supernatant column-purified. The S1 ran as a single peak on ion-exchange and gel filtration chromatography. All three light chains were present, and there was no sign of HMM in SDS-PAGE. F-actin, HMM and NEM-myosin were prepared by standard methods. To make the $1.1\text{-}\mu\text{m}$ polystyrene beads bind to F-actin irreversibly, they were coated with $1 \mu\text{g ml}^{-1}$ BSA-TRITC and $10 \mu\text{g ml}^{-1}$ NEM-treated monomeric myosin. Glass microspheres ($1.7 \mu\text{m}$ diameter) were applied to a coverslip as a suspension in 0.1% nitrocellulose; after drying, the coverslip was made part of

a 0.2-mm deep flow chamber and S1 was bound in a low ionic strength solution (in mM: 25 KCl , 10 Tris , pH7, 4 MgCl_2 , 1 EGTA). Rhodamine-phalloidin labelled F-actin and NEM-myosin labelled polystyrene beads were introduced in an ATP-containing *in vitro* motility assay buffer¹³ at 23°C . Two NEM-coated beads were captured by optical traps and an actin filament strung between them. The position of one bead was monitored by brightfield microscopy, using a four-quadrant photodetector². A glass microsphere (coated with S1 or HMM) was brought up close to the actin filament and the interaction with S1 or HMM observed. Each optical trap imposes a trap-stiffness κ_{trap} to its bead, the value of which can be set by varying the laser power. The actin filament stiffness in tension (as is the case in these experiments) is sufficiently great for the $10\text{-}\mu\text{m}$ lengths used¹⁴ that it can be considered rigid. The axial (*x*-axis) system stiffness (κ_x) is thus double the individual trap stiffness ($\kappa_x = 2 \kappa_{\text{trap}}$). When a myosin head attaches, its stiffness, κ_m , adds to provide a total stiffness $\kappa_{xm} = \kappa_x + \kappa_m$. The *y*-axis stiffness κ_y will, under all conditions, be approximately equal to κ_{trap} . The mean square deviation from the rest position in the absence of acto-myosin interaction is related to the system stiffness ($\langle x^2 \rangle = kT/\kappa_x$; $\langle y^2 \rangle = kT/\kappa_{\text{trap}}$)¹². The noise seen on the traces is close to that predicted from the independently derived value of κ_{trap} (Fig. 1*e, f*) and displays a Boltzmann distribution with half-width at half-height $(\ln 2) 2kT/\kappa_x^{1/2} \approx 12 \text{ nm}$ for the trap stiffness we have used (see below; Fig. 2*a–d*). κ_{trap} was calibrated using a bead with no actin filament attached (when $\kappa_x = \kappa_y = \kappa_{\text{trap}}$) by three methods. First, with the trapped bead held $5 \mu\text{m}$ from the coverslip a large amplitude triangle wave motion was applied to the microscope slide and Stokes' force² ($6\pi\eta av$, with viscosity η , bead radius a , stage velocity v) produced a bead movement proportional to trap compliance. Second, the mean square brownian motion was measured ($\langle x^2 \rangle$) over several seconds at 2.5 kHz bandwidth and the equipartition principle applied ($\frac{1}{2} \kappa_{\text{trap}} \langle x^2 \rangle = \frac{1}{2} kT$) (ref. 12). Third, the most convenient check of trap stiffness was by on-line power-spectrum analysis using the 3-dB roll-off in brownian noise spectrum ($v_c = \kappa/2\pi\beta$; ($\beta = 6\pi\eta a$)¹²; this was measured $5 \mu\text{m}$ from the coverslip surface. Using this method, laser power was adjusted until data matched a predetermined lorentzian curve. Four-quadrant detector sensitivity was determined by applying a known displacement to the laser tweezer position in the *x*- and *y*-axes. When feedback was applied between the quadrant detector and the optical trap position, stiffness was raised to 5 pN nm^{-1} , measured by applying large Stokes' force. With an actin filament held taut between two trapped beads, κ_x was usually set to be between 0.03 and 0.05 pN nm^{-1} . This stiffness is sufficiently lower than that of the acto-S1 interaction (at least 0.17 pN nm^{-1} ; see Figs 1*e, f* and 3*c*) to allow the magnitude of the elementary movement to be estimated.

FIG. 2 *a, b*, Frequency distribution of the amplitude of displacements, A_x , scored by measuring displacements that were evident above background of residual brownian noise (*a*, 39 traces from 18 filaments; *b*, 73 traces from 21 filaments). The fits are Boltzmann distributions with $A_x = A_0 \exp \{ -\frac{1}{2} \kappa (x - x_0)^2 / kT \}$, in which x_0 is the displacement caused by crossbridge interaction. The arbitrary κ and amplitudes were fitted by assuming that all of the data belong to a single distribution in which values are missing for zero and small displacements (for fitting method see *e* below). The crossbridge work stroke, x_0 , of these fits is 3.5 nm for S1 and 5.0 nm for HMM. *c, d*, Frequency distribution of the amplitude of displacements measured for a more restricted data set (*c*, 9 traces from 4 filaments; *d*, 5 traces from 2 filaments) in which the system was sufficiently stable to estimate crossbridge attachments by the increase in system stiffness (fall in local variance). Using this method, zero and near-zero displacements were quantified (see Fig. 1*c, d*). The solid lines have the same values of κ and x_0 as in *a* and *b*, with A_0 scaled to fit the frequency of observations. The broken lines are the best-fit Boltzmann distributions to the data. For S1 the value of x_0 predicted from these data is 3.3 ± 0.73 (s.e.m.) nm and the value of κ is 0.052 pN nm^{-1} . For HMM, $x_0 = 7.9 \pm 0.91$ nm, with $\kappa = 0.031 \text{ pN nm}^{-1}$. Note that there are many observations with small or zero displacement. *e*, The data of *a* and *b* were recalculated as $\log(\text{frequency})$ versus $(x - x_0)^2$, where x is the observed displacement and x_0 is a constant, thereby causing both 'tails' of the distributions to be positive, and making normal distributions linear, giving a gradient proportional to κ_x and the y-intercept of $\log(A_0)$. For this calculation, frequencies of 0 or 1 were omitted, as were those for displacements between -7.5 and $+10$ nm (so only the tails of the histograms were used). The small graphs on the left-hand side show plots of $\log(\text{frequency})$ versus $(x - x_0)^2$ for three values of x_0 . The main figure shows the value of the regression coefficient of the best linear fit to this relationship versus x_0 . The parameters with the highest regression coefficients gave, for S1, $x_0 = 3.5$ nm, $\kappa = 0.046 \text{ pN nm}^{-1}$, $A_0 = 131$; and for HMM, $x_0 = 5.0$ nm, $\kappa = 0.047 \text{ pN nm}^{-1}$, $A_0 = 126$. Note that, in all cases, the fitted value of κ is very close to the value of κ_x for these experiments. *f, g*, Isometric forces from S1 and HMM; amplitude of each event was estimated by eye.

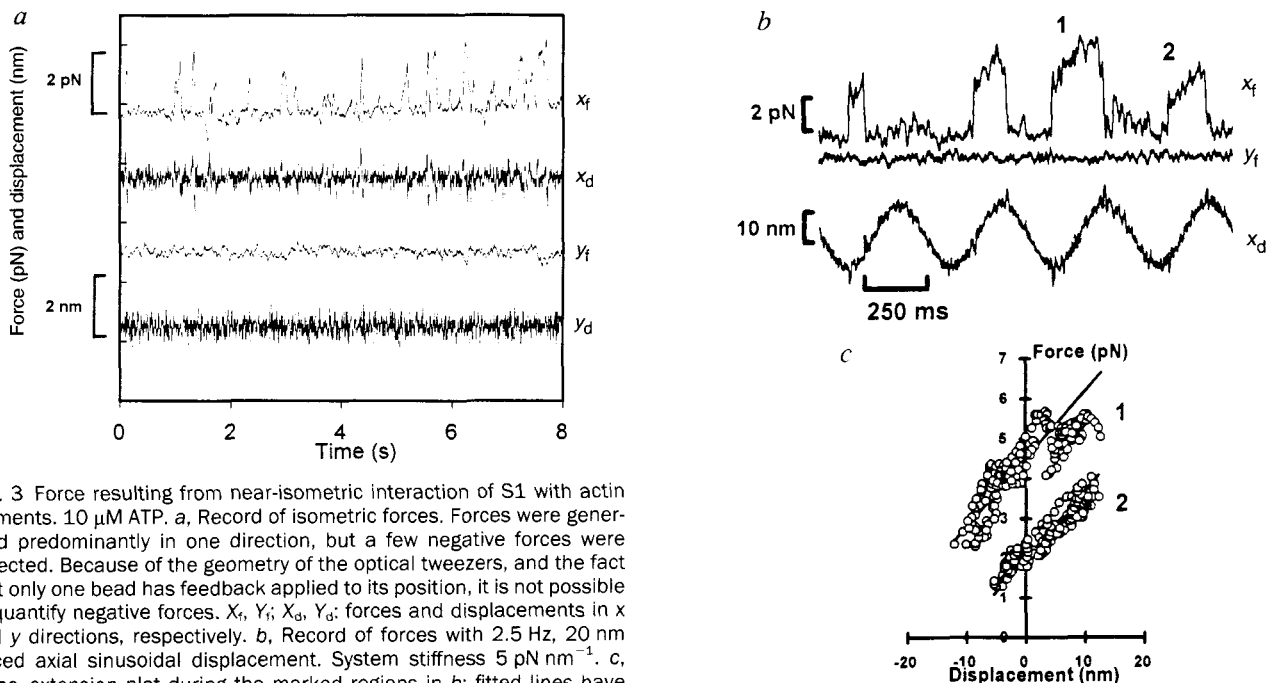
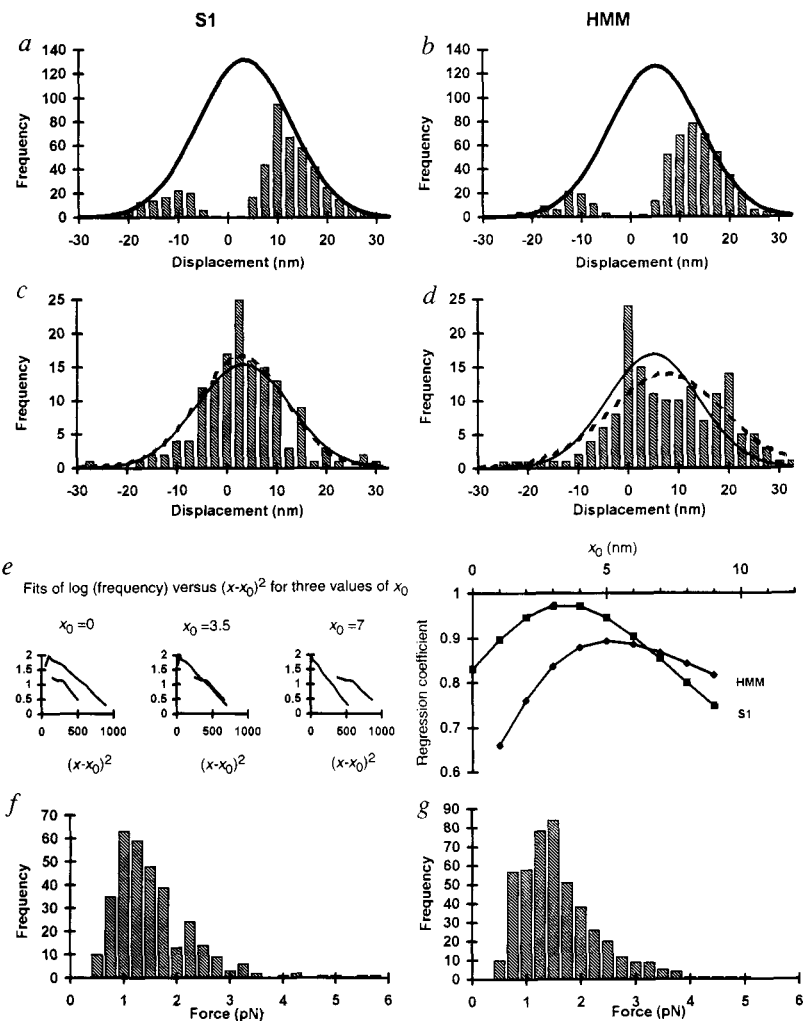


FIG. 3 Force resulting from near-isometric interaction of S1 with actin filaments. $10 \mu\text{M}$ ATP. *a*, Record of isometric forces. Forces were generated predominantly in one direction, but a few negative forces were detected. Because of the geometry of the optical tweezers, and the fact that only one bead has feedback applied to its position, it is not possible to quantify negative forces. X_f , Y_f ; X_d , Y_d : forces and displacements in x and y directions, respectively. *b*, Record of forces with 2.5 Hz , 20 nm forced axial sinusoidal displacement. System stiffness 5 pN nm^{-1} . *c*, Force-extension plot during the marked regions in *b*; fitted lines have a gradient of 0.17 pN nm^{-1} (mean value was 0.17 pN nm^{-1} , $N = 7$ filaments; 25 cycles total).

of displacements will thus have the same half-width as that of the brownian motion in the absence of attachments, but will be shifted by the size of the working stroke (x_0) from the mean rest position. The observed data can be fitted by such a distribution shifted by about 4–5 nm (Fig. 2a, b). However, observations at or close to zero displacement are missing because, in this and previous analyses^{2,3,5}, only displacements that deviate clearly from the mean rest position can be scored. The bimodal distribution is simply the two tails of a single broad distribution of crossbridge interactions (Fig. 2c, d). For S1 the data obtained by scoring attachments (Fig. 2c) fit closely to that predicted (solid curve of Fig. 2a). For HMM the fit is less good, and it is possible that both heads are contributing to the data observed.

We do not know the extent to which compliance of attachment between S1 and the nitrocellulose substrate acts as an elastic element during force generation; a consequence of this is that our estimates of force and stiffness are lower limits. However, unknown external compliance does not contribute to our estimate of displacement because this is determined from measurements at low force. In addition, the orientation of S1 or HMM on the substrate will presumably be random in these experiments. If the attached head head orientates itself with sufficient flexibility that it 'points' in the direction of the actin filament then the above values are correct; however, if the movement produced

is determined by its orientation with the substrate then the true value is determined by averaging a cosine term through 180°, which means that our values of both force and displacement will be underestimates by a factor of $\pi/2$ compared to those measured in muscle-fibre experiments¹¹. The finding that the myosin work-stroke size is close to the F-actin monomer repeat is intriguing. Similar findings have been made for kinesin, for which the work stroke seems to be close to the tubulin monomer repeat¹². □

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A potential catalytic site revealed by the 1.7-Å crystal structure of the amino-terminal signalling domain of Sonic hedgehog

Traci M. Tanaka Hall*, Jeffery A. Porter†, Philip A. Beachy† & Daniel J. Leahy*‡

* Department of Biophysics and Biophysical Chemistry, and † Howard Hughes Medical Institute, Department of Molecular Biology and Genetics, Johns Hopkins University School of Medicine, 725 North Wolfe Street, Baltimore, Maryland 21205, USA

WITHIN the past few years, members of the *hedgehog* (*hh*) family of secreted signalling proteins have emerged as the primary signals generated by certain embryonic patterning centres. In vertebrate embryos, for example, *sonic hedgehog* expression in the notochord appears to be responsible for the local and long-range induction of ventral cell types within the neural tube and somites (reviewed in refs 1, 2). Protein products encoded by *hh* family members are synthesized as precursors that undergo autoprocessing to generate an amino-terminal domain that appears to be responsible for both local and long-range signalling activities, and a carboxy-terminal domain that contains the autoprocessing activity^{3,4}. As part of an effort to understand how *hh* family members participate in cell-to-cell signalling, we have determined and report here the crystal structure at 1.7 Å of the amino-terminal domain of murine *Sonic hedgehog* (Shh-N)^{5–9}. The structure revealed a tetrahedrally coordinated zinc ion that appears to be structurally analogous to the zinc coordination sites of zinc hydrolases, such as thermolysin and carboxypeptidase A. This previously unsuspected catalytic site represents a distinct activity from the autoprocessing activity that resides in the carboxy-terminal domain.

Crystals of both native and selenomethionyl-substituted (SeMet) Shh-N were grown, and the structure of SeMet Shh-N

was determined by the multiwavelength anomalous diffraction (MAD) method^{10,11}, as summarized in Table 1. The Shh-N structure consists of a core $\alpha + \beta$ sandwich of two α -helices and a six-stranded, mixed β -sheet ($\beta 1$ – $\beta 6$) decorated by extensive loop regions. A small, two-stranded, antiparallel β -sheet (β' and β'') is also present. Ribbon drawings and a topology diagram of the Shh-N structure are shown in Fig. 1. This overall fold has not to our knowledge been observed in other proteins. A computer-assisted database search using the program DEJAVU¹² and an extensive search of the SCOP (Structural Classification of Proteins) database¹³ failed to identify any similar structures.

After a complete protein model of Shh-N had been built, the original experimentally phased electron-density maps revealed an unexplained peak of more than 11 times the standard deviation of the maps. His 141, Asp 148, His 183 and an apparent water molecule were arranged about this peak in a tetrahedral configuration, and separated from it by distances consistent with metal coordination (Fig. 2a). This arrangement is strikingly similar to the coordination of zinc ions in zinc hydrolases such as thermolysin and carboxypeptidase A (Fig. 2b), despite otherwise unrelated protein folds^{14,15}. In zinc hydrolases, a glutamic acid that is believed to abstract a proton from the zinc-bound water molecule has been shown to be essential for enzymatic activity¹⁶, and an analogous residue, Glu 177, is present in Shh-N. Owing to these similarities, we tested for and confirmed the presence of approximately stoichiometric amounts of zinc in *Escherichia coli*-expressed Shh-N by atomic absorption spectroscopy (data not shown). The structure of Shh-N suggests that the coordinated zinc has a catalytic function, as the zinc in Shh-N is exposed to solvent (Fig. 1b) and a water molecule is bound to the zinc in the crystal structure, rather than a fourth amino acid side chain. Structural zinc ions not directly involved in catalysis are generally sequestered from solvent and coordinated by four amino acid side chains that usually include cysteine¹⁶.

In addition to the zinc-coordinating residues and the essential glutamic acid, other residues in thermolysin and carboxypeptidase A are believed to assist in catalysis. An examination of the Shh-N structure reveals residues well positioned to function analogously in Shh-N. These residues include His 135, His 181 and Glu 127 of Shh-N. Figure 2c shows a schematic model of the Shh-N zinc site and illustrates how these residues might

‡ To whom correspondence should be addressed.

function in a hydrolytic cycle based on mechanisms for thermolysin and carboxypeptidase A¹⁶.

The amino acid sequences of the N-terminal domains of Hh family members are identical at 66–99% of amino acid positions (selected sequences are shown in Fig. 3), and the N-terminal domains of all *hh* proteins therefore are expected to share a similar structure. The conservation of residues near the zinc binding site is even greater, with zinc-coordinating histidine and aspartic acid residues absolutely conserved among vertebrate Hh sequences, and the non-coordinating residues thought to be important for catalysis almost absolutely conserved (the only exception is the rat, in which His 181 is replaced by arginine). It seems likely, therefore, that the N-terminal domains of these *hh* proteins coordinate zinc in a manner similar to Shh-N.

In striking contrast, the N-terminal domains of *hh* proteins in *Drosophila melanogaster*¹⁷ and *D. hydei*⁹ contain only one of the coordinating residues, His 141. Asp 148 is replaced by threonine, His 183 is replaced by tyrosine, and Glu 177 is replaced by valine. Thus the N-terminal domains of *Drosophila* proteins would not be expected to bind zinc or catalyse a hydrolysis

reaction in the same manner as those of vertebrates, despite an overall sequence match of 66%. This difference between the *hh* proteins of vertebrates and *Drosophila* does not simply reflect differences in vertebrates and invertebrates, as the zinc binding residues are retained in the mosquito, which, like *Drosophila*, is a dipteran (see also legend to Fig. 3). Three explanations seem plausible: the activity mediated by the zinc site is not utilized in *Drosophila*; *Drosophila* Hh achieves this activity in a different fashion; or the activity is performed by another molecule.

Crystal packing suggests that Shh-N might hydrolyse a peptide substrate. A crystal lattice interaction between two symmetry-related Shh-N molecules is shown in Fig. 4a. The carboxy terminus of molecule 1 is positioned at the zinc binding site of molecule 2 and shows how Shh-N could interact with a peptide product after hydrolysis. A stereo view of the contacts made near the zinc coordination site is shown in Fig. 4b. The main-chain atoms of Ala 194 and Lys 195 of molecule 1 (coloured purple in Fig. 4b) form several hydrogen bonds with residues in a crevice near the zinc coordination site of molecule 2. The lysine side chain also seems to fit well in a shallow pocket formed by

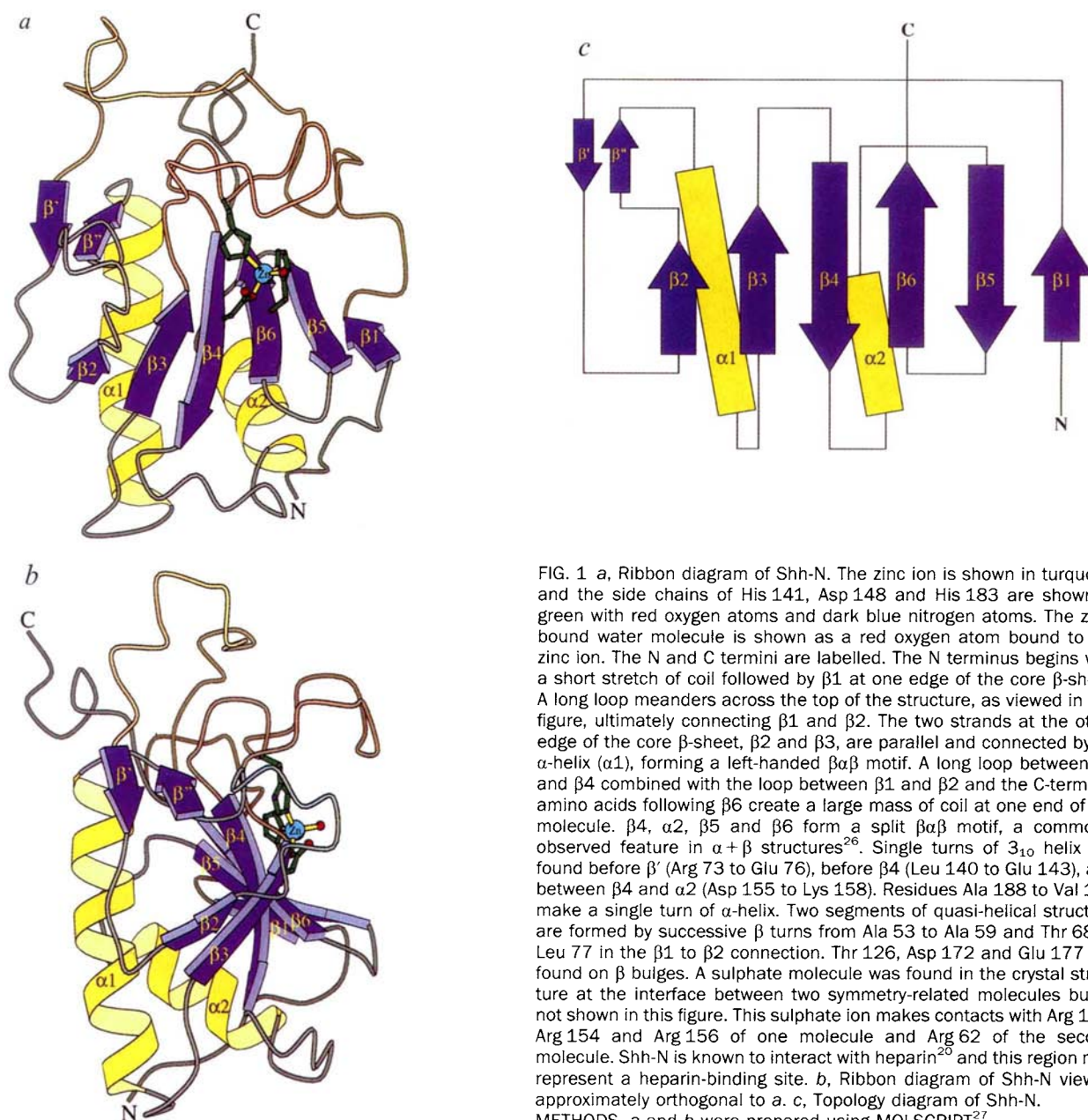


FIG. 1 *a*, Ribbon diagram of Shh-N. The zinc ion is shown in turquoise and the side chains of His 141, Asp 148 and His 183 are shown in green with red oxygen atoms and dark blue nitrogen atoms. The zinc-bound water molecule is shown as a red oxygen atom bound to the zinc ion. The N and C termini are labelled. The N terminus begins with a short stretch of coil followed by $\beta 1$ at one edge of the core β -sheet. A long loop meanders across the top of the structure, as viewed in this figure, ultimately connecting $\beta 1$ and $\beta 2$. The two strands at the other edge of the core β -sheet, $\beta 2$ and $\beta 3$, are parallel and connected by an α -helix ($\alpha 1$), forming a left-handed $\beta\alpha\beta$ motif. A long loop between $\beta 3$ and $\beta 4$ combined with the loop between $\beta 1$ and $\beta 2$ and the C-terminal amino acids following $\beta 6$ create a large mass of coil at one end of the molecule. $\beta 4$, $\alpha 2$, $\beta 5$ and $\beta 6$ form a split $\beta\alpha\beta$ motif, a commonly observed feature in $\alpha + \beta$ structures²⁶. Single turns of 3_{10} helix are found before β' (Arg 73 to Glu 76), before $\beta 4$ (Leu 140 to Glu 143), and between $\beta 4$ and $\alpha 2$ (Asp 155 to Lys 158). Residues Ala 188 to Val 192 make a single turn of α -helix. Two segments of quasi-helical structure are formed by successive β turns from Ala 53 to Ala 59 and Thr 68 to Leu 77 in the $\beta 1$ to $\beta 2$ connection. Thr 126, Asp 172 and Glu 177 are found on β bulges. A sulphate molecule was found in the crystal structure at the interface between two symmetry-related molecules but is not shown in this figure. This sulphate ion makes contacts with Arg 124, Arg 154 and Arg 156 of one molecule and Arg 62 of the second molecule. Shh-N is known to interact with heparin²⁰ and this region may represent a heparin-binding site. *b*, Ribbon diagram of Shh-N viewed approximately orthogonal to *a*. *c*, Topology diagram of Shh-N. METHODS. *a* and *b* were prepared using MOLSCRIPT²⁷.

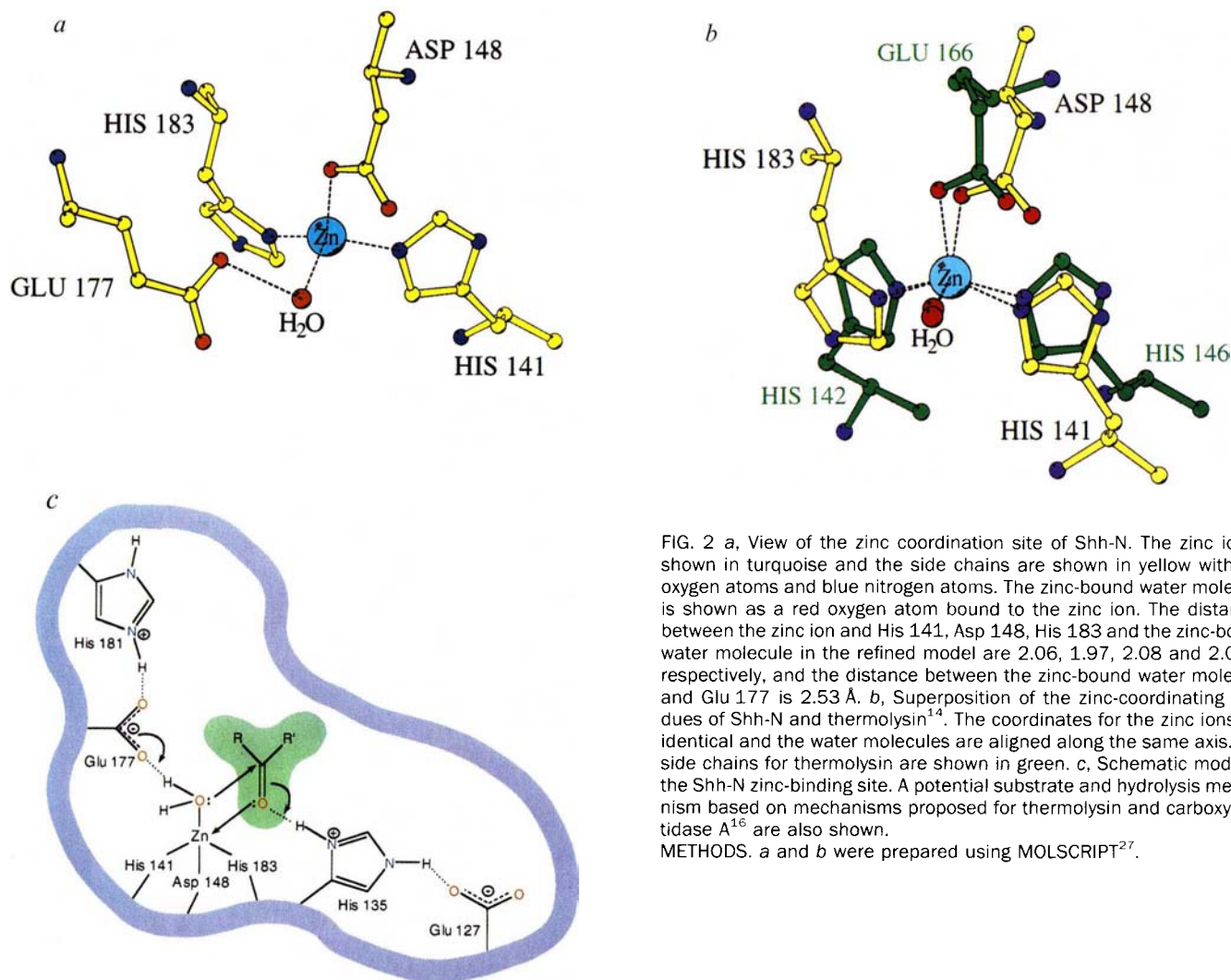
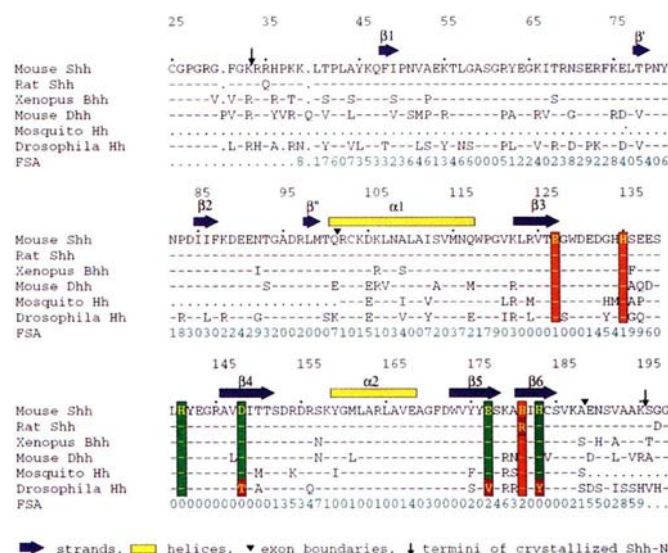


FIG. 2 *a*, View of the zinc coordination site of Shh-N. The zinc ion is shown in turquoise and the side chains are shown in yellow with red oxygen atoms and blue nitrogen atoms. The zinc-bound water molecule is shown as a red oxygen atom bound to the zinc ion. The distances between the zinc ion and His 141, Asp 148, His 183 and the zinc-bound water molecule in the refined model are 2.06, 1.97, 2.08 and 2.05 Å, respectively, and the distance between the zinc-bound water molecule and Glu 177 is 2.53 Å. *b*, Superposition of the zinc-coordinating residues of Shh-N and thermolysin¹⁴. The coordinates for the zinc ions are identical and the water molecules are aligned along the same axis. *c*, Schematic model of the Shh-N zinc-binding site. A potential substrate and hydrolysis mechanism based on mechanisms proposed for thermolysin and carboxypeptidase A¹⁶ are also shown.

METHODS. *a* and *b* were prepared using MOLSCRIPT²⁷.

FIG. 3 Amino acid sequence alignments of Hh N-terminal domains. Sequences of murine Shh-N⁹ (the sequence in ref. 6) differs by one amino acid residue from that published in ref. 6), the N-terminal domains of rat Sonic hh⁸, *Xenopus* Banded hh (Bhh)²⁸, mouse Desert hh⁶, mosquito Hh⁹ and *Drosophila* Hh¹⁷ are shown. The amino-acid sequence of Shh-N is shown with homologous sequences aligned below. Residues identical to the murine sequence are indicated by dashes. Numbering is for murine Shh-N. Residues involved in the zinc coordination site and the catalytic glutamic acid are highlighted with green. Coordinating residues and the catalytic glutamic acid that are identical in other Hhs are also highlighted with green. Non-coordinating residues likely to be important for catalysis are highlighted with orange. Non-identical active site residues are highlighted with red. Partial sequences of hh proteins from the flour beetle, *Tribolium castaneum*, the leech, *Hirudo medicinalis*, and the sea urchin, *Strongylocentrotus purpuratus*⁹ (not shown) indicate that at least two of the coordinating residues (His 141 and Asp 148) and two of the non-coordinating residues (Glu 127 and His 135) are conserved (the only exception is *S. purpuratus* which has an asparagine in place of His 135). The secondary structure assignments for Shh-N obtained using the algorithm in ref. 29 are shown. Fractional solvent accessibility (FSA) is shown in green for each residue in the Shh-N structure. The FSA is the ratio of the solvent-accessible surface area of a residue in a Gly-x-Gly tripeptide to that in the Shh-N structure. A value of 0 represents a value from 0.00 to 0.09, 1 represents 0.10 to 0.19, and so on.



Phe 48, Trp 173 and Tyr 175, suggesting that Shh-N might cleave its own C terminus between Lys 195 and Ser 196. The zinc-bound water molecule (coloured green in Fig. 4b) is positioned adjacent to the terminal carboxylate of Lys 195, as if Shh-N had hydrolysed the peptide bond between Lys 195 and Ser 196 and was preparing for another cycle of hydrolysis. Furthermore, approximately 1370 Å² are buried at the intermolecular interface between the symmetry-related molecules in the crystal lattice, and the surfaces appear to be complementary in shape and charge. However, attempts to demonstrate proteolytic activity in Shh-N with a peptide encompassing the C terminus of Shh-N (Glu 189 through to Gly 198) have so far been unsuccessful. Gel filtration further indicates that Shh-N is a monomer in solution (data not shown). Additional studies will be necessary to determine whether conditions can be found under which Shh-N displays proteolytic activity.

The discovery of a potential hydrolytic activity in Shh-N raises the questions of what its substrate might be, and what role it might play in the function of Shh-N and its homologues. An enticing hypothesis is suggested by the observation that Hh N-

terminal domains become tethered to the cell membrane by an activity residing in the C-terminal domain (J.A.P. and P.A.B., unpublished results) thus restricting the N-terminal domain for local signalling; intermolecular autoproteolysis of the type suggested by the crystal structure might then release Shh-N for long-range signalling, as appears to occur in the induction of sclerotome and possibly motor neurons^{1,2}. Our inability to demonstrate autoproteolysis of Shh-N *in vitro* does not rule out the existence of this activity *in vivo*.

In *Drosophila*, the loss of zinc-coordinating residues in Hh-N would imply a loss of this intermolecular autoproteolytic activity and a consequent loss of the ability to be released for long-range signalling. This loss would be consistent with the demonstration that long-range patterning by Hh of the wing imaginal disc is indirect, through induction of the decapentaplegic signalling protein, and the further suggestion that other forms of long-range signalling might also be indirect¹⁸.

Other possible functional roles for Shh-N hydrolase activity include activation of a receptor by proteolysis, as in the case of thrombin¹⁹, cleavage and subsequent activation of other poly-

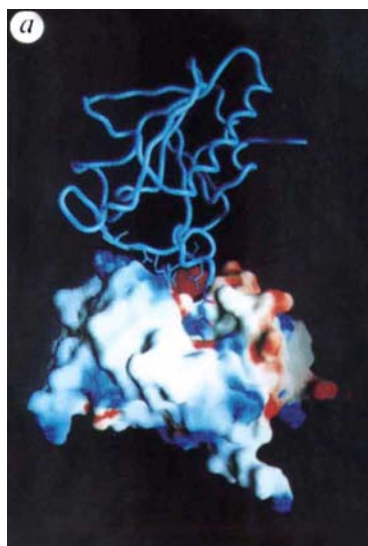


FIG. 4 a, Crystal lattice interaction between two symmetry-related molecules of Shh-N. One molecule is pictured as a backbone trace (molecule 1) and the other (molecule 2) is shown with the molecular surface area coloured by electrostatic potential. Positively charged regions are coloured blue and negatively charged regions are coloured red. Side chains of molecule 1 that are buried as a result of contact are shown in blue. Ala 194 and Lys 195 of molecule 1 are shown in purple with nitrogen atoms coloured blue and oxygen atoms coloured red. b, Stereo view of the crystal lattice interaction near the zinc coordination site. Molecule 2 is shown with a transparent surface, coloured as in a. Side chains His 141, Asp 148, Glu 177 and His 183 of molecule 2 are shown in yellow. The zinc ion is shown in turquoise. The zinc-bound water molecule is shown as a green oxygen atom. METHODS. This figure was prepared using GRASP³⁰.

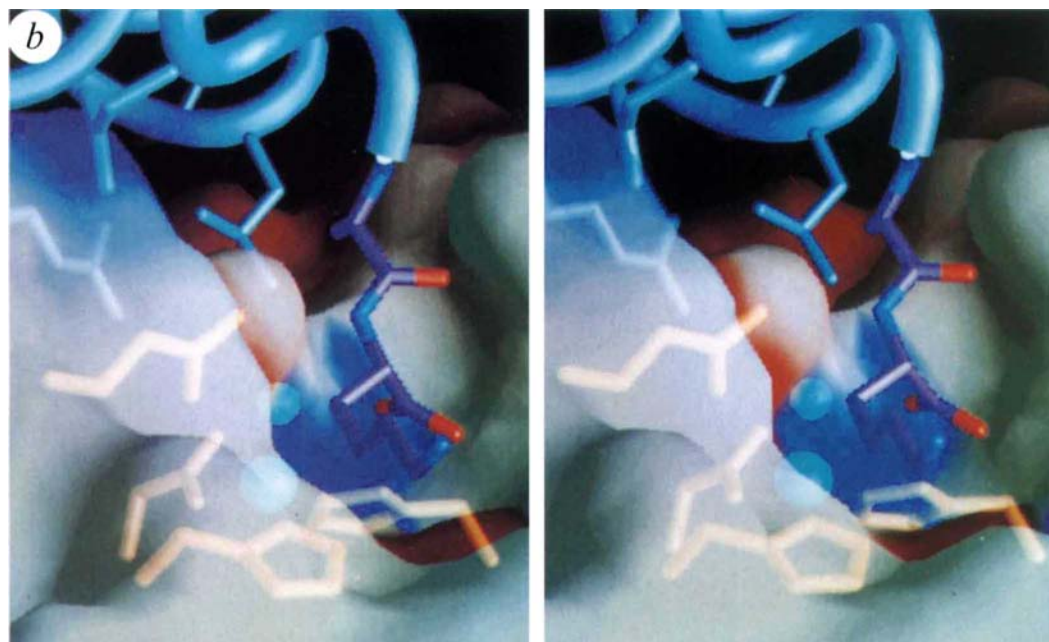


TABLE 1 Statistics for data collection, phase determination and refinement

(a) Data collection statistics (30.0–1.7 Å)

Wavelength (Å)	Reflections (N)	Redundancy	Completeness (%) [*]	Signal (<I/σI>)	R _{sym} (%)
0.9879	14,837	6.7	85.7 (98.0)	13.1	7.3
0.9793	14,968	6.7	86.4 (97.9)	12.8	7.8
0.9792	15,069	6.7	87.0 (98.4)	12.9	8.2
0.9686	15,105	6.7	87.2 (97.9)	12.7	8.0

(b) MAD structure factor ratios and anomalous scattering factors

Wavelength (Å)	0.9879	0.9793	0.9792	0.9686	f' (e)	f'' (e)
0.9879	0.047	0.058	0.056	0.056	-4.13	0.51
0.9793		0.059	0.041	0.062	-9.35	3.67
0.9792			0.072	0.059	-8.42	5.12
0.9686				0.062	-3.86	4.18

(c) Refinement and stereochemical statistics

R-value	0.191 (F > 2σ, 6.0–1.7 Å)	0.202 (all F, 6.0–1.7 Å)
Free R-value	0.261 (F > 2σ, 6.0–1.7 Å)	0.273 (all F, 6.0–1.7 Å)
Average B (Å ²)	10.53 for protein, 24.54 for solvent	
R.m.s. deviations		
Bonds (Å)	0.009	
Angles (deg)	1.97	
B-values (Å ²)	1.51/1.56 bonds/angles of main chain	
	2.77/2.98 bonds/angles of side chains	

Protein expression and purification. Native Shh-N protein from Cys 25 to Gly 198 was produced as a glutathione S-transferase fusion protein in *E. coli* strain BL-21 (DE3), cleaved with thrombin, and purified as previously described²⁰. SeMet Shh-N protein was expressed in *E. coli* strain B834 (DE3), a methionine auxotroph, as previously described²¹ and purified in the same manner as native protein using degassed buffers throughout the procedures. The N terminus of the protein crystallized was Arg 34 as determined by Edman degradation. The C terminus was Lys 195, as determined by mass-spectrometric analysis of cyanogen bromide-cleaved fragments. Analysis of Shh-N purified under different conditions demonstrated that formation of both the N and C termini was dependent on prolonged incubation in the presence of trace amounts of thrombin. **Crystallization.** Crystals were grown from hanging drops by vapour diffusion. A 3.4 mg ml⁻¹ solution (2 μl) of Shh-N in 1.4 mM β-mercaptoethanol was mixed with 1 μl of a 1:1 dilution of reservoir solution (30% PEG 8000, 0.12M ammonium sulphate, 10 mM sodium cacodylate, pH 6.5) with distilled water and equilibrated over the reservoir solution. Diffraction-quality crystals of SeMet protein were only obtained when hanging drops were prepared under anaerobic conditions. Crystals typically grew to a final size of 0.8 × 0.3 × 0.02 mm over 5–20 days. Crystals are in space group P2₁2₁2₁ with unit cell dimensions: a, 53.7; b, 79.0; and c, 35.4 Å. **Data collection and processing.** All data were collected from crystals cryopreserved in buffer containing the mother liquor and 20% (w/v) ethylene glycol and flash frozen in a gaseous nitrogen stream at -180 °C. Diffraction data were collected from native and SeMet crystals with an R-axis Ilc detector and CuKα radiation from a Rigaku RU200 rotating anode. MAD data were collected at four wavelengths from a single SeMet crystal at beamline X-4A of the National Synchrotron Light Source at Brookhaven National Laboratory. Data were collected using Fuji HR-V phosphorimaging plates and digitized using a Fuji scanner. Oscillations of 2° at φ and φ + 180° were collected with no overlap for each oscillation range at each wavelength. All diffraction images were processed using the program DENZO and scaled with the program SCALEPACK²². <I+> and <I- > were used for MAD phase determination and partially recorded reflections were used in all cases. Diffraction data from different wavelengths were scaled with WLSCL and optimal f' and f'' were calculated with MADLSQ²³. **Structure determination.** Three selenium sites were deduced from difference Patterson maps of SeMet versus native data sets. MAD phase determinations were made with the program MLPHARE and solvent-flattening and histogram-matching were performed with the program DM²³. The experimental electron-density map was computed using MAD-derived phases for reflections from 20.0 to 1.8 Å and in most regions proved of comparable detail to final (2F_o - F_c) e²/e² maps. An atomic model consisting of Leu 40 to Ala 194 was readily built into 1.8-Å electron-density maps using the program 'O'²⁴. One round of simulated annealing and several rounds of Powell minimization with X-PLOR²⁵ alternated with model building with 'O' yielded the current model of Shh-N consisting of 157 residues, Lys 39 to Lys 195; 1 zinc ion, 1 sulphate ion, and 120 water molecules. All refinements were carried out using the 0.9793-Å SeMet data. No electron density was observed for residues 34–38. One molecule is present in the asymmetric unit, and the solvent content is approximately 35%. All backbone torsion angles are within energetically acceptable regions. (a), R_{sym} and completeness values were calculated considering Bijvoets equivalent. R_{sym} = 100 × Σ_i |I_i(h) - <I(h)>| / Σ_i I_i(h). (b), R.m.s. (Δ|F|)/r.m.s. (|F|) where ΔF is the Bijvoet difference at one wavelength (values on the diagonal) or the dispersive difference between two wavelengths (values off the diagonal). Also shown are the anomalous components of the Se scattering factors as a function of wavelength as determined by MADLSQ²³. (c), A subset of the data (5%) were excluded from refinement and used for the free R-value calculation²⁶. All data for which |F| > 2σ were used in the refinement. R-value = Σ_i |F_o| - |F_c| / Σ_i |F_o|.

^{*} Values in parentheses are percentage completeness to 2.0 Å.

peptide signalling molecules, control of local pH in a manner analogous to carbonic anhydrase, or hydrolysis of membrane lipids to initiate signal transduction in a manner similar to phospholipase C, another zinc hydrolase. Insight into the substrate and functional role of an Shh-N hydrolase might be gained by assessing the signalling capacities of Shh-N mutants lacking the residues likely to be involved in any hydrolase activity. □

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ERRATUM

Glacial-interglacial changes in nutrient utilization in the equatorial Pacific Ocean

J. W. Farrell, T. F. Pedersen, S. E. Calvert & B. Nielsen

Nature **377**, 514–517 (1995)

FIGURES 1 and 2 in this Letter in the 12 October issue were transposed during the production process. The figure legends are correct. □

Neuroscience solutions

This supplement for neuroscience technology describes postmitotic human neurons, a new microscope for three-dimensional illumination, neurophysiology simulation software, antibodies for neurobiological research and a CCD camera.

THE analysis of brain tissue samples is said to be simplified by the Intracel **brain slice chamber**, which is designed to maintain isolated living tissue *in vitro* (Reader Service No. 100). This permits stable electrophysiological recordings to be made from the preparation. Both interface and submerged methods of slice maintenance are



The Intracel brain slice chamber — to maintain isolated living tissue *in vitro*.

accommodated. The machined acrylic chamber incorporates a low-noise heater, and permits the height of the perfusion fluid to be easily adjusted. Versions are available for heating and cooling above and below ambient temperatures. The medium is preheated or precooled by the system, before contact with the tissue. The system consists of the chamber, together with a controller with an LCD temperature read-out.

Medical Systems has announced the development of the D180 isolated stimulator, specially designed to provide brief electrical pulses required to **stimulate the cerebral cortex through the intact scalp**, or to stimulate the spinal cord percutaneously



Medical Systems' cerebral stimulator.

(Reader Service No. 101). The D180 provides single, very brief, high-voltage pulses and features a low-output impedance. In this way, the cortex can be stimulated without undue discomfort; the method has also been successful in stimulating the spinal cord. Safeguards to protect the subject include use of an output isolating transformer and a limitation on the maximum rate of pulse generation. The D180 is intended for research purposes.

Human hNT neurons from Stratagene are fully **postmitotic human central nervous system (CNS) cells** that can be produced in large quantities in the laboratory (Reader Service No. 102). Derived from Ntera2/D1 (NT2) committed neuroepithelial precursor cells, these cultured hNT neurons offer neuroscience researchers the

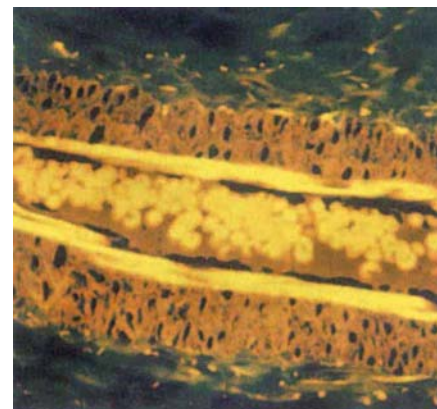
opportunity to study a human neuronal system without the need to obtain scarce human primary tissue. The company now offers eight new cDNA libraries derived from NT2 precursor cells and hNT neurons at two distinct stages in the process of differentiation to mature postmitotic neurons. The NT2 cell lines are induced by retinoic acid (RA) to differentiate *in vitro* into postmitotic t95 neurons. These cells also permit HIV infection. Stratagene also offers these new cDNA libraries derived from uninucleated NT2 precursor cells, NT2 cells after 24-hour RA induction, hNT neurons after 6-week differentiation and hNT neurons after 12-week differentiation. Each of the four libraries was constructed in both the UniZAP YR vector and ZAP Express vector *EcoRI/XhoI* vector. These powerful λ -ZAP vectors provide automatic *in vivo* excision or cloned fragments. In addition, the ZAP Express vector offers both prokaryotic and eukaryotic expression.

Imaging systems

The new Bio-Rad model GS-525 molecular imager and new model GS-505 molecular imager systems are said to represent an innovative approach to storage **phosphor imaging of radioactive and chemiluminescent samples** (Reader Service No. 103). With either a PC or Macintosh computer, the

systems are designed to quickly scan and accurately quantify virtual images and sample up to 35×43 cm without the need for X-ray film, intensifiers, or low-temperature film storage and exposure. Three different screen chemistries in either 20×25 cm or 35×43 cm sizes, are available for a variety of sample types, including ^{32}P , ^{33}P , ^{125}I , ^{14}C , ^{35}S , tritium and chemiluminescent labelled gels and blots.

Data Cell offers the **calcium image-ratio system**, a fully integrated system of software and hardware (Reader Service No. 104). By monitoring the distribution of calcium concentration, nerve transmission, muscle contraction and other cellular reactions can be objectively measured. Fluorescent markers are used to identify different chemical elements, which are selectively measured in intensity using spectral filters. Because of the speed of such reactions, real-time hardware is



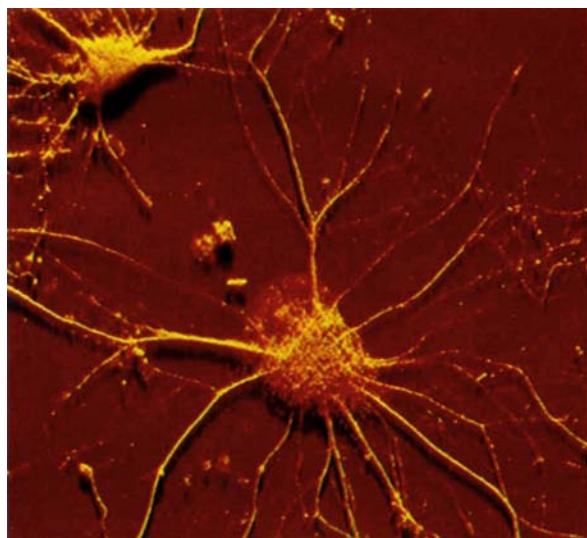
Data Cell calcium image-ratio system.

used to capture the changes and return calcium distribution and permeability values. Experiments can be recorded and played back for further viewing or analysis. The system includes image acquisition and processing hardware, an automated filter wheel and a choice of intensified or cooled cameras. The system is provided completely integrated under a custom Windows interface.

The Edge R400 features patented Multiple Oblique illumination, which generates separate left- and right-eye views for **real-time, true-colour, three-dimensional observation** directly through the eyepieces (Reader Service No. 105). The three-dimensional images can be photographed as true-colour, three-dimensional pairs or single-picture three-dimensional

sional anaglyphs and acquired by video for computer image processing and quantitative analysis. By adjusting the intensity and obliqueness of the four independent illuminating beams, users can obtain a better image. This is true whether a specimen is viewed and recorded in three dimensions or in two. It also can offer an efficient and cost-effective alternative to scanning techniques for three-dimensional imaging. Its capabilities include common techniques and preparations, such as the examination of thick slices, including brain slices, live specimens and explant cultures, in addition to brain mapping, the ability to view interrelationships among nerve processes. For example, observers can follow dendritic pathways and determine the orientation of structures in three-dimensional space. For micromanipulation the large and accessible stage accommodates direct mounting of a micromanipulator. The three-dimensional view facilitates targeting probes along the z-axis and verifies penetration. In patch-clamping techniques, the high resolution and contrast facilitate accurate cell-surface contact.

Meridian Instruments has introduced the Insight Point **laser-scanning confocal microscope** (Reader Service No. 106). The microscope is said to allow direct ocular



Meridian Instruments' Insight Point laser-scanning confocal microscope.

previewing of samples, and capture of multi-colour, point-scanned confocal images with a CCD camera. User-friendly software and computer automation are designed to make image acquisition easy.

Molecular means

The **human DNA identification kit** from Boehringer Mannheim was specifically assigned to simplify the identification of human sequences in hybrid cells that contain only a part of the full chromosomal complement from a donor human cell

(Reader Service No. 107). The kit contains all the enzymes, nucleotides and buffers needed selectively to amplify human target sequences that lie between repetitive sequences in the *Alu* family, by inter*Alu* PCR. The human DNA identification kit contains *Alu* PCR primers (CL1 and CL2), which allow amplification of all the DNA between two *Alu* repeat sequences while amplifying only a very short part of the repeat sequences themselves. Also included in the kit is control, hybrid-cell DNA, which contains human chromosome 16 DNA as the sole human component and which can be used as a positive control in the inter*Alu* and *in situ* hybridization reactions.

TCS Biologicals offers a comprehensive **range of antisense oligonucleotides**, which is designed to block the expression of a specific target gene, resulting in a depletion of the respective protein product (Reader Service No. 108). Subsequent loss of function analysis allows a rapid and detailed study of the physiological or pathological role of the inhibited gene. Manufactured by Biognostik, all oligonucleotides are purified by a proprietary six-step process (including HPLC), quality checked and supplied with an individual batch analysis. Products are priced according to amount actually delivered, rather than by synthesis scale. As well as a large selection of antisense oligonucleotides, the range also includes an antisense starter kit and FITC- and BrdU-labelled antisense oligonucleotides for cellular uptake studies. Also offered are Hybriprobe unmodified DNA oligonucleotides as hybridization probes for Southern and northern blots or *in situ* hybridizations. Control oligonucleotides and a custom synthesis service are also available.

The Access **RT-PCR system** from Promega is a new two-enzyme, single-tube system for PCR amplification using an RNA model as the starting template (Reader Service No. 109). Avian myeloblastosis virus reverse transcriptase (AMV RT) is used for first-strand cDNA synthesis. After generation of this DNA, amplification is performed using heat-stable *Tfl* DNA polymerase. In the optimized buffer provided with the system, AMV RT is active at 48 °C. This high reaction temperature is used during reverse transcription to enhance further the ability of the enzyme to process even the most difficult RNA templates. Unlike single-enzyme systems, this system is designed to require no addi-

tions to the reaction mix between the reverse transcription and amplification step. This reduces both the hands-on time of the protocol and the risk of contamination of the sample. The system is said to be extremely sensitive and can detect a 540-bp, β -actin RT-PCR product in as little as 1 pg of total RNA after 40 cycles.

Serva now offers a precast gel kit for **electrophoretic separation of cerebrospinal fluid** samples and isoelectric focusing (IEF) of proteins contained in cerebrospinal fluid (CSF) (Reader Service No. 110). This is an established method for screening patient samples for the presence of immunoglobulins (IgG), indicative to positive diagnosis of multiple sclerosis. The oligoclonal IgG shows up as multiple bands in the pH region of 7–10. The Servalyt Precotes CSF kit provides five



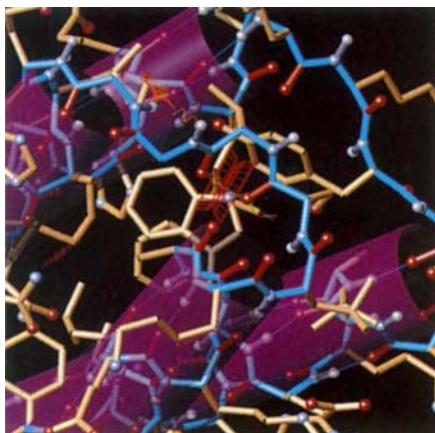
Serva precast gel kit for CSF separation.

precast polyacrylamide gels of the pH range 3–10, ready to use, and the other components needed to perform horizontal IEF of spinal fluid (buffers, wicks and applicator strip). Two different gel formats are available: 245 × 125 mm for routine processing of samples and 100 × 100 mm for the Sartorius IEF chamber. Each batch of gels is quality-control checked applying focusing of CSF samples. An optimized protocol for silver staining comes with the kit, ensuring high-sensitivity detection of unconcentrated samples.

For efficient **enterokinase cleavage** Invitrogen offers EnterokinaseMax (EKMax), a recombinant preparation of the catalytic subunit of enterokinase (Reader Service No. 111). Less than one unit of this highly purified enzyme is said to cleave 20 μ g of a typical fusion protein cleanly and efficiently. The manufacturer states that each lot of the product is stringently tested to ensure the absence of nonspecific proteases and efficient cleavage of fusion proteins.

Software

Interactive Simulations has announced the release Sculpt, an interactive **molecular modelling application** that embodies a radically new approach to computer-based molecular modelling (Reader Service No. 112). The program allows the



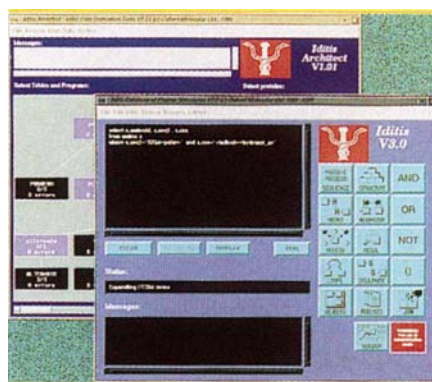
Sculpt by Interactive Simulations.

user to grab onto atoms, secondary structures and ligands, and move them with a mouse, while other atoms move due to the changed atom's position. Designed to be easy to use for many tasks, it should prove useful for exploring alternative side-chain packing, determining how an active site accommodates a ligand, designing proteins and peptides *de novo*, packing secondary structures and understanding molecular behaviour. The program currently runs on a PowerMac with 16 megabytes of memory and the Silicon Graphics workstation running IRIX 5.2 or later.

Neurosim, the **neurophysiology simulation program**, from Biosoft has now been released as a Windows package (*Reader Service No. 113*). The user first selects the experimental and neurophysiological parameters desired for the particular simulation and then runs the experiment. The computer generates a graphic display similar to that of an oscilloscope in genuine electrophysiological experiments. The parameters can then be varied to explore the effects of differing conditions. A wide range of phenomena can be simulated, including refractory period, threshold accommodation, voltage-clamp and single-channel patch-clamp. Various drugs can be applied, and the temperature and ionic concentrations can be varied. 'Network' allows the user to construct arbitrary circuits of neurons interconnected by non-spiking or spiking chemical synapses, or electrical synapses. Many different types of synapses can be defined, including chemical synapses with different reversal potentials, synaptic strengths and facilitation properties and electrical synapses with different rectification properties. The membrane properties of each neuron can be set individually, including the option of making a neuron an endogenous burster. Tonic or random input with defined characteristics can impinge on any neuron. 'Patch' simulates the kinetic properties of single ion channels. Three simple models are supplied: a two-state open/shut chan-

nel, a three-state agonist-activated channel (shut/unbound, shut/bound, open/bound), and a three-state shut, open and blocked channel. The program can also model a channel with up to five states with user-defined, transition rate constants. Open-time and shut-time histograms can be displayed, also with multiple-exponential curves superimposed.

Oxford Molecular Group has launched Iditis 3.0, a new version of the company's **protein structure database system** (*Reader Service No. 114*). This new version stores over 500 fields of data for each published protein structure derived from the latest Protein Data Bank (PDB) release in a series of efficient relational tables. This uses only 60 per cent of the hard disk space of the equivalent PDB release. Molecular



Oxford Molecular Group's Iditis software.

and structural biologists can now recreate PDB files exactly, byte-for-byte, eliminating the need to store separate copies of the PDB files. This efficient management of protein structure data allows users to perform in-depth structural analysis at nearly twice the speed of the previous release. Iditis runs on UNIX workstations and is used to study structural similarities in related protein families. This information should prove useful for designing an enzyme inhibitor, examining natural variations in structures of active sites on proteins and analysing tolerances to form folds in proteins.

Antibody detection

The Indirect kit from NEN Life Science Products is designed to be a simple, rapid, ultrasensitive system for the **detection of small amounts of antigen** (*Reader Service No. 115*). The manufacturer states that signals up to 1,000 times stronger can be achieved with TSA-Indirect versus standard chromogenic or fluorescence detection. The kit is based on a horseradish peroxidase protocol that catalyses the deposition of numerous biotins at the original antigen site. Visualization with streptavidin conjugates, either enzyme or fluorescently labelled, bound to the biotin completes the reaction. As the enhance-

ment of the signal is extremely specific, the signal-to-noise ratio is significantly improved. The entire reaction is said to take less than 10 min. Fluorescent strept-avidin conjugates, fluorescein, Texas red or coumarin, optimized for TSA-Indirect, can also be purchased separately.

Upstate Biotechnology has developed a new range of **antibodies and related reagents for neurobiology research** (*Reader Service No. 116*). These latest additions to the company's established product ranges for cell-signalling research are available from TCS Biologicals. Of particular interest for researchers working in the neurobiology field are a variety of antibodies to calcium, sodium and potassium channels, glutamate receptor antibodies and an antibody that recognizes NCAM (CD56) and can be used to recognize tumours of neuroectodermal origin by immunocytochemistry.

Chemicon has announced that a **rabbit polyclonal antibody to NR2** is now available (*Reader Service No. 117*). The manufacturer states that *N*-methyl-D-aspartate (NMDA) receptors play a key role in the central nervous system and have been shown to be directly involved in neuronal development, synaptic plasticity, learning and memory. The NMDA receptor complex in mammalian brain is made up of two types of subunits, NR1 and NR2. Four distinct NR2 subunits, NR2A, NR2B, NR2C and NR2D, have been identified. The NR2 subunits are not functional by themselves, but combine with the NR1 subunits to produce a functional receptor complex. Expression of the NR2 subunits changes during development and varies with brain region. The antibody is affinity purified and can be used for immunocytochemistry (both light and electron microscopy), western blotting and immunoprecipitation.

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PRODUCT REVIEW

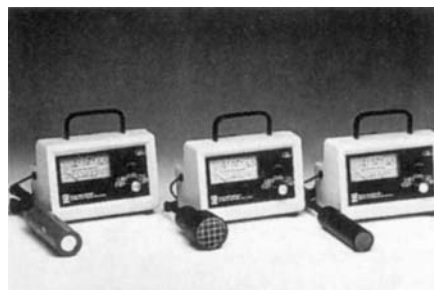
Oncogene-Calbiochem has introduced **c-fos peptide-specific antisera** specifically developed and screened for the ability to stain floating rat brain tissue sections (*Reader Service No. 118*). Implicated in cell growth, differentiation and development, this immediate early gene is induced by a large number of stimuli. Induction of the 62K c-fos protein is rapid but transient and represents about 0.1 per cent of the total cellular protein. The fos protein associates with the 39K c-jun protein and this complex binds specifically a sequence element in DNA referred to as the AP-1 binding site. Large lot sizes and rigorous calibration are said to minimize lot-to-lot variation. The product is supplied as hyperimmune rabbit serum. Detailed protocols are provided for both acrolein- and paraformaldehyde fixed tissues.

Odds and ends

Neurochemicals for the Neuroscientist is a catalogue produced by Research Biochemicals International, a supplier of pharmacologically active compounds (*Reader Service No. 119*). Items from this line of neurochemicals include excitatory amino acids, dopaminergics, PET/SPECT reagents, signal transduction agents and neuropeptides. New products just introduced include NMDP (nicotinate adenine dinucleotide phosphate), a novel activator of intracellular Ca^{2+} release,

which has been shown to act through an $InsP_3$ and cyclic ADP ribose-independent mechanism, and epibatidine, an alkaloid excreted from the skin of the poison arrow frog, which is said to be 200 times more powerful than morphine as an analgesic.

The Rad-Monitor **radiation/contamination detection instruments**, from Research Products International, is designed to monitor contamination from



Rad-Monitors for radiation detection.

radioisotopes, such as ^{32}P , ^{14}C , ^{35}S , ^{45}Ca , ^{131}I and ^{125}I (*Reader Service No. 120*). Three models are available: the model GM1 has a general purpose detector, the model GM2 has a high-sensitivity, wide-area detector and the model SD10 features a scintillation probe for detecting ^{125}I . The instruments are designed for use in a laboratory environment — a

corrosion-proof plastic housing resists damage from common solvents and acids. Monitors feature a logarithmic-scale, adjustable trip alarm, internal speaker, low-battery indicator, tube-saturation indicator and a five-position selector control. Models GM1 and GM2 also have a microrads per hour scale for dose-rate measurements.

Recent developments in the 4100 series of **cooled CCD cameras** from Astromed have allowed studies of voltage-sensitive dyes to be made for neurological applications, according to the manufacturer (*Reader Service No. 121*). Improvements in camera readout rates are said to enable changes resulting from electrical activity in brain tissue to be monitored. The dyes used for these applications alter their absorbance characteristics as a function of applied voltage. These changes can be quite small and need to be detected in the presence of a large background signal. The 4100 series of cameras has a dynamic range of 4,192 grey levels compared with 256 grey levels for a conventional camera. Sub-array pixel sampling has increased the image readout rates for these cameras, allowing faster events to be followed. □

These notes are compiled by Brendan Horton from information provided by the manufacturers. For more details, fill in the reader service card bound inside the journal.

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